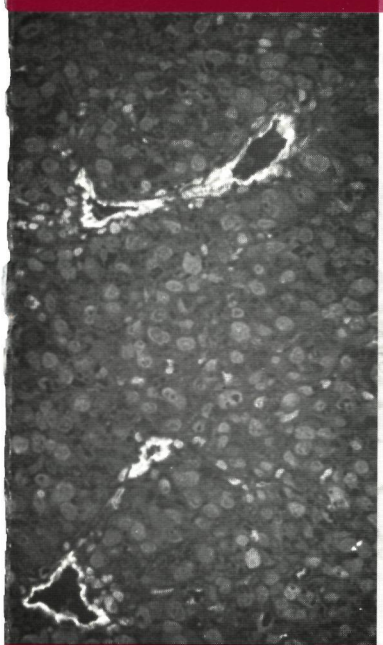
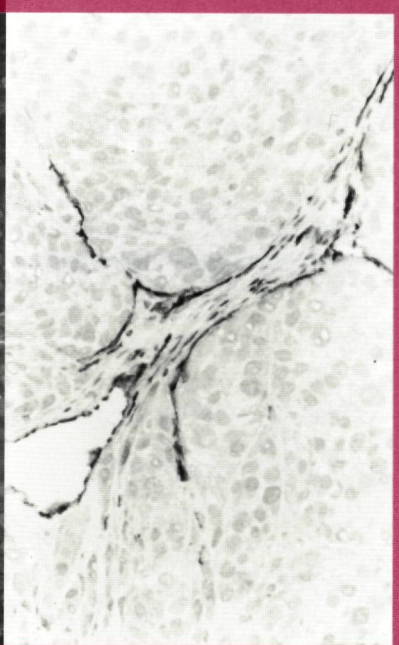
Vascular permeability factor, a key regulator of endothelial cell function and tumour angiogenesis





Andy Pötgens

Vascular permeability factor, a key regulator of endothelial cell function and tumour angiogenesis

Cover picture: Sections of melanoma xenografts in nude mice stained with a monoclonal antibody specific for mouse vascular endothelium. A tumour from the human melanoma cell line Mel57 stably transfected with vector DNA only is shown on the left, and a tumour from line Mel57 transfected with a VPF expression construct is shown on the right. Magnification: 200x.

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a key regulator of endothelial cell function

and tumour angiogenesis

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CIP

The studies presented in this thesis were performed at [1]the Institute of Pathology of the University Hospital Nijmegen, and at [2]the Department of Molecular Biology of the Catholic University Nijmegen, The Netherlands, under supervision of Prof. Dr. D.J. Ruiter[1], the late Prof. Dr. J.G.G. Schoenmakers[2], Dr. R.M.W. de Waal[1], and Dr. N.H. Lubsen[2]. The work was financially supported by the Netherlands Cancer Society (NKB) grant 90-02.

Ing haffel jeluk
is mieë weëd
wie ing kaar versjtank.

Voor Pa en Ma,
en voor Simone

Contents:

Chapter 1

General Introduction

A modified version of this Chapter is in press in:
Biological Chemistry Hoppe-Seyler.

1.1 Tumour angiogenesis

The process of angiogenesis

One of the major roles of the vasculature is its transport function: the supply of oxygen and nutrients to cells and the transport of waste products from cells. In higher organisms blood vessels are essential for the survival of nearly every living tissue. The initial formation of the vasculature in embryonic development is called vasculogenesis, and involves the differentiation of blood vessels from mesoderm tissue and from blood islands in the yolk sac. The growth of new vessels from existing blood vessels is called angiogenesis or neovascularization. In adult organisms most blood vessels do not grow: angiogenesis only takes place in regenerating tissues like the female reproductive organs or during wound healing. Angiogenesis is a highly regulated process involving many stimulatory and inhibitory factors (for a review see 1,2, and below).

Tumour angiogenesis

Tumour cells of course are as dependent on the vasculature as cells in normal tissues. Avascular tumours do not grow beyond the size of 1-2 mm^3, whereas after vascularization tumours quickly expand to much larger sizes (3). Mitotic rates of tumour cells decrease with increasing distance from capillaries (3) and tumours with a high blood supply have a higher fraction of dividing cells and lower necrosis rates than tumours with a low blood supply (4). Thus, tumours are truly dependent on angiogenesis for their own growth. Furthermore, angiogenesis is essential for metastasis, the most life-threatening aspect of cancer, as blood vessels provide an escape road for disseminating tumour cells (1,3,5). Many studies have recently been published which report a correlation between tumour vascularization and metastatic risk in patients (6-10).

Apart from the fact that angiogenesis is essential for the growth of tumours themselves, tumour vasculature is also important in clinical practice. The response to anti-tumour therapies, like conventional chemotherapy, as well as e.g. the use of photodynamic sensitizers, or of anti-tumour antibodies coupled to toxins or radionuclides, is largely dependent on the presence, distribution and permeability of tumour blood vessels (11-15). Of course, also in tumour imaging the delivery of tracers depends on the degree of tumour vascularization.

Properties of the tumour vascular bed

Much effort has been put into studying the vasculature of tumours. To distinguish blood vessels in cross-sections of tissues or tumour specimens, markers are used which stain one of the components of the vascular system. There is a number of polyclonal antibodies reacting with endothelial cell-associated antigens, such as von Willebrand factor (vWF) or angiotensin-converting enzyme (16), as well as monoclonal antibodies directed against

endothelial cells (17,18). Also some lectins, e.g. from Ulex europaeus or Grifonia simplicifolia (19), are used to stain endothelial cells. These markers are generally not completely specific for endothelial cells, and do not recognize all types of endothelium (reviewed in 20). Antibodies reacting with pericytes (21,22) or with components of the basal lamina (e.g. laminin or collagen type IV (16)) can be used as vascular markers as well. Again, these markers also cross-react with other cell types, or with extracellular matrix from other cell types (20). For this reason, in studying the vasculature of tumours, or of any tissue, several vascular markers should be used in parallel to obtain highest reliability.

Tumour angiogenesis has properties somewhat different from normal angiogenesis: the fast growth of tumour cells requires that angiogenesis takes place at a high rate in order to supply all parts of an expanding tumour. Although different types of tumour vasculature exist (23), a series of structural abnormalities is often found in tumour vessels, when compared with blood vessels in normal tissues. In general, tumour vasculature is considered to be of an "immature" nature. Some of the characteristics often reported to be found in tumour vasculature are: a high turnover rate of endothelial cells, gaps in the endothelial lining and basement membrane, low amounts of pericytes, a heterogeneous (or chaotic) organization and distribution of the tumour vasculature (the existence of e.g. non-functional capillary sprouts, or arteriovenous anastomoses), an increased vascular permeability, and the occurrence of intravascular coagulation (reviewed in 1,11,24, and 23,25). Tumour blood flow, oxygen pressure, and tissue pH values are generally very variable, but on average mostly lower than in many normal tissues (11). Because of the absence of lymphatic vessels, interstitial pressure is often high in tumours, leading to further transport problems (15). It is not surprising therefore that necrosis is a general phenomenon, especially in larger tumours (4,11).

Intravascular coagulation in tumour vessels

An experimental approach for anti-tumour therapy with the vasculature as its target, is the induction of intravascular clotting in the tumour vascular bed, thereby obstructing tumour blood flow. Within normal blood vessels there is a non-thrombogenic environment, due to heparin-like molecules and other anti-coagulant molecules on the endothelial lining (26). However, tumour vasculature is often prone to intravascular coagulation, which leads to spontaneous deposition of fibrin and the occurrence of intravascular clots or thrombi (25).

Malignant cells themselves can produce procoagulant molecules like tissue factor and "cancer procoagulant" (27,28) and provide a cell surface on which thrombin formation can be completed (29), but this only leads to fibrin formation outside of the tumour vessels. Intravascular clotting in tumour blood vessels is likely to be caused by soluble factors secreted by tumour cells that induce the expression of coagulation factors on neighbouring endothelial cells. Cytokines like interleukin-1 (30), vascular permeability factor (31), endothelial-monocyte activating polypeptide II (EMAP II, 32,33) and some

less well-characterized proteins (34,35) induce the procoagulant molecule tissue factor on endothelial cells, and have been shown to be expressed by tumour cells.

Tumour necrosis factor (TNF) also is an inducer of tissue factor on endothelial cells, and, when administered to mice carrying Meth A fibrosarcomas, TNF caused an elevated deposition of fibrin, appearance of thrombi and a reduced blood flow, specifically in the tumour vascular bed, ultimately leading to tumour necrosis (36). Apparently, the tumour vasculature was more sensitive to TNF treatment than the vasculature in normal tissues. This may be caused by tumour-produced factors which act synergistically with TNF in tissue factor induction. Such factors, "Meth A factor" (or EMAP I), vascular permeability factor, and EMAP II have indeed been identified in conditioned media from these sarcoma cells (31,35,33). Treatment of human cancers with TNF, either by direct administration, or indirectly, as achieved in the past after exposure to "Coley's toxins", which induce the endogenous expression of TNF, has had varying success. Recently, however, some trials have been reported in which human sarcomas, melanomas, and squamous cell carcinomas were treated successfully with TNF in combination with other agents (e.g. interferon-γ and melphalan) in isolated limb perfusions (37-40). The remissions resulting from such treatments were accompanied by signs of activation of tumour endothelial cells, platelet aggregation, and coagulative and haemorrhagic necrosis.

To understand the mechanisms that lead to intravascular coagulation in the tumour vasculature, either spontaneously or after TNF treatment, it is important to identify the factors produced by human tumour cells that induce this effect. In purification protocols of such factors (31,35), an *in vitro* assay which measures induction of tissue factor on endothelial cells is generally used. Induction of tissue factor activity was preceded by an elevation of tissue factor mRNA levels in the endothelial cells (31). Tissue factor induction can therefore also be assayed at the RNA level. A quantitative RT-PCR assay might facilitate the measurement of tissue factor mRNA levels, and therefore might aid the search for procoagulant-inducing factors, as it would require only small amounts of endothelial cell RNA to be tested. The development of such an assay will be described in Chapter 2 of this thesis.

1.2 Angiogenic factors

Positive regulation of angiogenesis is mediated by molecules referred to as "angiogenic factors", and in order to induce the growth of blood vessels, tumour cells have to produce such angiogenic factors. In general, angiogenic factors belong to the classes of growth factors or cytokines, but it should be noted that the action of angiogenic growth factors is distinct from the action of growth factors involved in oncogenic transformation, the process of cells becoming independent of external stimuli for cell division. Growth factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor alpha (TGFα), fibroblast growth factors (FGF), and many more, are often expressed by tumour cells along with their cognate receptors, which can lead to autocrine

stimulation of tumour cell growth. It is not surprising therefore that the genes of some growth factors and receptors have initially been identified as (proto-)oncogenes (for instance the PDGF-B gene: c-*sis*, the FGF-3 gene: *int-2*, the EGF-R gene: c-*erbB*; reviewed in 41,42). The mode of action of angiogenic factors, on the other hand, is paracrine, i.e. the target of the growth factor is another cell type than the producing cell.

The angiogenesis-inducing effect of growth factors is usually tested in a chicken chorioallantoic membrane (CAM) assay or in a corneal micropocket assay (1), but in some cases growth factors are being considered angiogenic only because of their ability to promote endothelial cell division. The angiogenic factors mentioned in this Chapter are summarized in Table 1.

Angiogenic factors that stimulate endothelial cell division

Many angiogenic factors directly stimulate the growth of endothelial cells. Among the first factors characterized as angiogenic factors are: acidic and basic fibroblast growth factor (aFGF and bFGF, the first one was also known as endothelial cell growth factor: ECGF, 43-45), transforming growth factor alpha (TGFα), and a factor related to TGFα, epidermal growth factor (EGF, 46). These factors are often produced by tumour cells and they have a direct stimulating effect on endothelial cell mitosis. These factors also have an effect on a variety of other cell types, however, and can play a role in transformation of tumour cells as well (reviewed in 41,42). Platelet-derived growth factor (PDGF) is another pleiotropic mitogen which is also considered an angiogenic factor, since it induces cellular proliferation of human microvascular endothelial cells (47).

Some other angiogenic factors have a more restricted target cell specificity, like platelet-derived endothelial cell growth factor (PD-ECGF), vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), placenta growth factor (PlGF, a factor closely related to VPF/VEGF), and interleukin 8 (IL-8). PD-ECGF is an inducer of *in vivo* angiogenesis, and has been found to be produced by tumour cells (48,49). Its growth stimulatory effect on endothelial cells has been doubted, however: a stimulatory effect of PD-ECGF on DNA synthesis in endothelial cells has been demonstrated, but no effect on cellular proliferation has been reported (2). Interesting in this respect is the finding that PD-ECGF is related to, if not identical with thymidine phosphorylase, an essential enzyme in nucleic acid metabolism (50). VPF/VEGF does stimulate endothelial cell division *in vitro* as well as angiogenesis *in vivo*, and is gaining more and more interest, as it is clear that it plays an essential role in tumour angiogenesis (51-53). PlGF also stimulates proliferation of endothelial cells, and is therefore considered an angiogenic factor. PlGF has been found to be produced by some tumours (54,55). IL-8 is a cytokine mainly secreted by macrophages, and is an inducer of endothelial cell proliferation and of angiogenesis *in vivo* (56). Recently, it was shown that IL-8 is produced by melanoma cells and that the level of IL-8 expression correlated with the metastatic potential of the melanoma lines in nude mice (57).

Angiogenic factors

Factor	Effect on EC proliferation	EC migration	EC proteolytic activity
aFGF	+	+	+
bFGF	+	+	+
TGFα	+		
EGF	+		
VPF/VEGF	+	+	+
PlGF	+		
PD-ECGF	+/0	+	
IL-8	+	+	
PDGF	+/0		
TGFß	-	-	-
TNFα	-	+	
Angiogenin	0	0	

Table 1. Growth factors and cytokines that stimulate angiogenesis. Their effects on endothelial cells *in vitro* are shown. Symbols: +, stimulatory effect; -, inhibitory effect; **0**, no effect; if the effect is not known, no notification is given. Angiogenic factors that do not stimulate endothelial cell division, migration, or proteolytic activity are believed to induce angiogenesis indirectly. For abbreviations and references see the text.

Indirectly acting angiogenic factors

Some factors which are angiogenic *in vivo* have no influence on endothelial cell proliferation *in vitro*, or even inhibit endothelial cell proliferation. These factors may induce angiogenesis indirectly by attracting and activating other cell types, like monocytes, and thus stimulate the production of directly acting angiogenic factors by these cells. Tumour necrosis factor alpha (TNFα) and transforming growth factor beta

(TGFß) belong to this class of molecules: they are angiogenic *in vivo* (58,59) but inhibit endothelial cell mitosis *in vitro* (60-65). Angiogenin is also angiogenic *in vivo* (66), but the mechanism by which it induces angiogenesis is somewhat enigmatic: it stimulates endothelial cells to increase 1,2-diacylglycerol and inositol triphosphate levels and to secrete prostacyclin (67,68), but it appears to have no effect on endothelial cell proliferation (1,2). Its major *in vitro* activity seems to be its ribonucleolytic activity towards ribosomal RNA, and thus inhibition of protein synthesis (69,70), which at first glance is difficult to reconcile with induction of angiogenesis. PDGF-BB is also thought to have an indirect effect on tumour angiogenesis, by promoting connective tissue stroma formation, which in its turn is a support for ingrowing blood vessels (71).

The importance of proteolysis and cooperation between angiogenic factors

It should be stressed that angiogenesis is a process involving more steps than endothelial cell proliferation alone. In order to form new vessels, endothelial cells have to migrate in a certain direction and form tubules. VPF/VEGF, aFGF, bFGF, IL-8, PD-ECGF, and TNFα are chemotactic for endothelial cells (72,73,44,45,48,56,58), and in that way contribute to migration. Breakdown of the basal lamina surrounding the endothelial cell layer is another important (and initial) step in migration. In endothelial cells bFGF and VPF/VEGF induce the expression of specific proteases involved in extracellular matrix degradation, like collagenases (44,45,74) and constituents of the plasminogen activator pathway. bFGF induces both urokinase plasminogen activator (uPA) and its inhibitor PAI-1, thereby increasing the uPA/PAI-1 ratio (75,76), while VPF/VEGF is most potent in the induction of tissue-type plasminogen activator (tPA, 76). In connection with these activities, bFGF and VPF/VEGF are capable of inducing invasion and capillary-like tube formation of endothelial cells in collagen gels (also called *in vitro* angiogenesis); at least bFGF does so for fibrin gels as well (77). VPF/VEGF and bFGF act synergistically in inducing *in vitro* angiogenesis in collagen gels (77), which may be explained by the fact that bFGF upregulates the expression of the receptors for VPF (78).

VPF activity might be suppressed by TNFα, since TNFα down-regulates the expression of VPF receptors (78). TGFß, which enhances the formation of extracellular matrix (79), modulates the effect of bFGF on the proteolytic activity of endothelial cells, by decreasing the uPA/PAI-1 ratio that is increased by bFGF (75). In *in vitro* angiogenesis in fibrin gels, however, TGFß either potentiates or inhibits the effect of bFGF, depending on the concentration of TGFß added (80).

It is of importance to note that some of the angiogenic factors (like TGFα and TGFß) are expressed as latent precursor molecules which are activated by specific proteolytic cleavage (81,82,79). Other angiogenic factors (the members of the FGF family, and some variants of VPF/VEGF) are immobilized by binding to heparin-like molecules in extracellular matrix, but are released by proteolytic processes (83,84). Thus, by inducing extracellular proteolysis angiogenic factors may activate or mobilize themselves or each other.

These examples indicate that the mode of cooperation of the many angiogenic factors *in vivo* is very complex. Some angiogenic factors may not be able to initiate all steps required for angiogenesis, but in concert with other factors can contribute to the completion of the whole process. Furthermore, the action of angiogenic factors is counteracted by several anti-angiogenic factors (85, see below), in normal situations leading to a balance between angiogenesis and anti-angiogenesis. This balance can shift towards angiogenesis if this is required by the situation, like for instance after wounding, during the menstrual cycle, or during tumour growth.

Anti-angiogenesis

A new and promising approach in anti-tumour therapy is the use of anti-angiogenic agents. The rationale behind this is that by blocking angiogenesis a tumour will become deprived of its nutrients and oxygen, and will eventually decrease in size or disappear. The mode of action of anti-angiogenic agents differs: they usually inhibit endothelial cell division, either directly, or indirectly by influencing the action of angiogenic factors. Anti-angiogenic factors often also influence the proteolytic properties of the endothelial cell surface (86).

Some endogenous, mutually unrelated molecules are inhibitors of angiogenesis, such as thrombospondin, platelet factor 4, a tissue inhibitor of metalloproteinase: TIMP (85), interferon-α (87) and an oestrogen metabolite (88). These factors probably play a role in maintaining the natural balance between stimulation and inhibition of angiogenesis. Also analogues or derivatives of endogenous molecules, products derived from bacteria or fungi, and synthetic molecules have been shown to be potent inhibitors of angiogenesis in experimental situations, e.g. retinoic acid, suramin (86), heparin (89), heparin-steroid conjugates (90), heparinase (91), fumagillin derivatives (92), and thalidomide (93). A growing list of angiogenesis inhibitors have also been shown to be effective suppressors of tumour angiogenesis and tumour growth (88,90,92,94-96), and at least one analogue of fumagillin, TNP-470, was successful in inhibiting metastasis of melanoma in mice (96).

An exciting discovery was recently made that primary tumours of a Lewis lung carcinoma subline produce an anti-angiogenic factor, called angiostatin, which is identical to an internal part of plasminogen. This tumour-derived factor suppresses angiogenesis in and growth of its own metastases (97). This factor, and other angiogenesis suppressors, might become of great therapeutic value. Perhaps even more inhibitors of angiogenesis will be discovered or developed in the future. A quest for inhibitors or receptor antagonists of angiogenic growth factors, for instance, might open interesting new possibilities.

1.3 Introducing VPF

Biological activities of VPF

In recent years, increasing attention has been paid to the angiogenic factor VPF/VEGF. Vascular permeability factor (VPF) was initially isolated as a protein factor secreted by guinea pig tumours growing in the peritoneal cavity, which induced accumulation of serum protein-containing ascites fluid. The isolated protein also induced rapid and transient leakage of serum proteins from the dermal vasculature in guinea pig skin without inducing endothelial cell damage, as measured in a so-called Miles assay (98). Later this factor was found to be identical to vascular endothelial growth factor (VEGF) or vasculotropin, which had been isolated and cloned by several independent groups as an endothelial cell-specific mitogen and an inducer of angiogenesis *in vivo*, as measured in a chorioallantoic membrane (CAM) assay (51,99-102). In the remainder of this Chapter, this factor will be referred to as vascular permeability factor (VPF), since its effect on vascular permeability (in guinea pig skin) is more pronounced than its effect on endothelial cell proliferation (84,98,101,103,104).

VPF has later been shown to induce rapid von Willebrand factor release from endothelial cells (105), to be chemotactic for endothelial cells (72), and to increase the expression of GLUT-1 glucose transporter (106), tissue factor (31), interstitial collagenase (74), plasminogen activators, and plasminogen activator inhibitor-1 (76) by endothelial cells (see Table 2). VPF thus induces multiple processes directly involved in angiogenesis, like endothelial cell proliferation, migration, and specific protease expression, but VPF also stimulates the activation of coagulation pathways by the endothelium and enhances vascular permeability. The latter phenomena are considered to be properties of tumour vasculature (11,24), but angiogenesis during wound healing is associated with hyperpermeable microvessels and formation of fibrin as well (107).

VPF is also capable of inducing endothelial cells to invade three-dimensional collagen gels and to form capillary-like tubes in it (77). VPF therefore has the capacity to promote angiogenesis in an *in vitro* model system on its own, although in cooperation with bFGF the induction of *in vitro* angiogenesis is strikingly more prominent (77).

VPF is often referred to as an endothelial cell-specific factor, but this is not completely true, as VPF also induces tissue factor expression on monocytes, and is chemotactic for monocytes (31). In all other cell types tested, VPF did not elicit a response, so it is probably correct to say that VPF has a very restricted target cell specificity, with endothelial cells being the main target.

Bioavailability of VPF

The VPF translation product is provided with a secretory signal peptide that is cleaved off during secretion from the producer cells (51,101). This feature is not common to all growth factors: for instance aFGF, bFGF and PD-ECGF lack a signal peptide, so in order

Biological effects of VPF

On cultured endothelial cells:

Immediate effects:
- Tyrosine phosphorylation
- Increase of intracellular Ca^{2+} and IP_3
- Release of vWF

Increased expression of:
- GLUT-1 glucose transporter
- Tissue Factor
- tPA, uPA, PAI-1
- Interstitial collagenase

Other effects:
- DNA synthesis and proliferation
- Chemotaxis
- Invasion and tube formation in collagen gels

***In vivo*:**

Immediate effect:
- Vascular permeability

Long-term effect:
- Angiogenesis

Table 2. Biological activities of VPF on endothelial cells *in vitro* and *in vivo*. For references and abbreviations see the text.

to reach their target cells alternative mechanisms of secretion must exist for these factors (108,48). But even with this secretory pathway, it is not self-evident that VPF can reach its target cells, since some variants of VPF are retained by the producer cells at extracellular sites.

Several molecular variants of the VPF protein exist due to alternative splicing of its mRNA. The pre-mRNA for VPF contains 8 exons, of which exons 6 and 7 can either be present or absent in the mature mRNA (109). The VPF variants generally found in human cells have a length of 189 amino acids (encoded by all 8 exons), 165 amino acids (lacking the amino acids from exon 6) and 121 amino acids (lacking the amino acids from exons 6 and 7, see Fig. 1). The analogous mouse, rat, and bovine proteins are 188, 164 and 120 amino acids long (99,110,111). Two other, rare forms of human VPF mRNA have been described in literature: a variant encoding a 206 amino acid protein (containing all exons and part of the 7^{th} intron) was found in liver (112), and a variant encoding a 145 amino acid protein (lacking exon 7 only) was found in uterine tissue (113).

Exon 6 of the VPF messenger codes for a basic stretch of amino acids, due to which the VPF splice variants containing these residues (VPF_{189}, VPF_{206}, and probably also VPF_{145}) have a high affinity for heparin (VPF_{189} elutes from heparin sepharose columns between 0.9-2.0 M NaCl). Because of this these variants are retained by the producer cells on extracellular heparin-like molecules, probably proteoglycans (84). VPF_{189} was only released from the cells after addition of suramin or heparin to the medium, or by heparinase treatment of the producer cells. Moreover, plasmin cleavage resulted in the release of the N-terminal half of VPF_{189}, which was still biologically active (84).

VPF_{165} has a lower affinity for heparin (it is completely eluted from heparin sepharose by 0.9 M NaCl), and is only partly retained by the producer cells, whereas VPF_{121} has no significant affinity for heparin and is completely released from the producer cells (84). These two soluble VPF variants are almost the only species found in conditioned media from mammalian cells, generally migrating as bands of 15-24 kD on SDS-PAGE under reducing conditions (114). Under non-reducing conditions, however, VPF migrates as a broad band of between 34 and 43 kD (98), indicating that in its native form VPF is a dimeric protein.

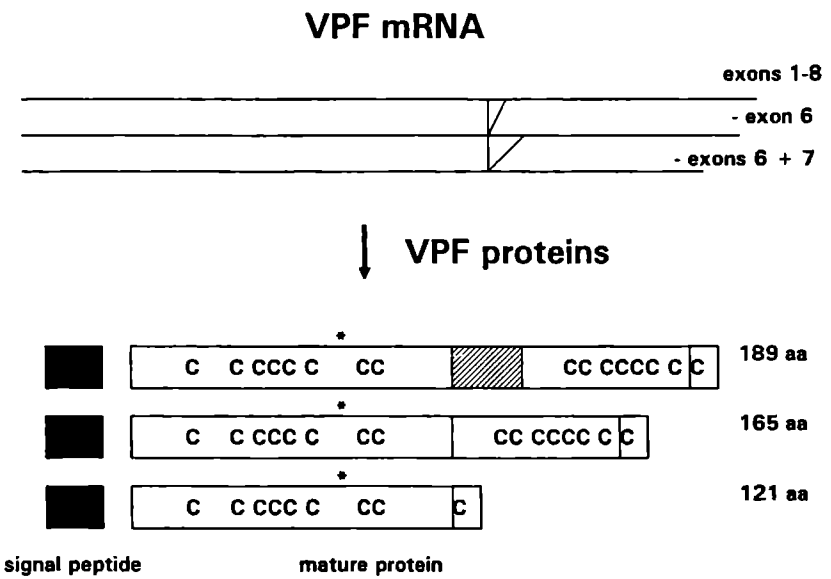

Figure 1. Schematic illustration of the three most abundant splice variants of VPF mRNA, and of the primary structures of the encoded proteins. *Solid boxes:* secretory signal peptides; *Hatched box:* highly basic amino acid stretch encoded by exon 6. Also depicted are the N-linked glycosylation site (*), and the cysteine residues (C). The eight cysteine residues in the N-terminal half of VPF are conserved with PDGF, the other cysteines are unique for VPF.

The amino acids encoded by exons 6 and 7 do not appear to be essential for biological activity (84), but as demonstrated above, they are important for the availability of VPF. The smaller VPF variants are diffusible molecules probably able to reach their target cells easily, while the variants containing the exon 6-encoded amino acids are stored extracellularly, but can be recruited, for instance by plasmin cleavage, if their action is needed. Specific expression and storage of cell-bound VPF variants in theory might be valuable for tissues which do not require instant angiogenesis. However, in most human tissues, tumours, and cultured cells - transformed or non-transformed -, the mRNAs coding for the soluble VPF variants of 121 and 165 amino acids were the predominant species (112,113,115-117). Also in embryonic and adult mouse tissue, and in rat endothelial cells the mRNAs for the soluble variants were the major species found (110,111). Exceptions were human kidney and haemangioblastoma, in which the VPF_{189} mRNA was expressed in abundancy along with the VPF_{121} mRNA (118), and rat heart, in which the VPF_{188} messenger was the predominant species (119).

Cellular receptors for VPF

In initial searches for VPF receptors endothelial cells of various sources were used, and two classes of VPF binding sites were always found. There were large differences in the affinities reported for these receptors, however. High affinity binding sites had dissociation constants of between 1-13 pM (with 500-25000 binding sites per cell), whereas for lower affinity binding sites dissociation constants of between 10-1500 pM were reported (with 3000-180000 sites per cell) (120-123,103). After cross-linking of VPF and receptor, complexes were detected of a molecular mass of 270 kD or 225 kD, often together with several smaller complexes. Binding of VPF to its receptors was inhibited by protamin and suramin (121,123), but was enhanced by heparin at concentrations between 0.1-10 μg/ml (124). Pretreatment of endothelial cells with heparinase abolished binding of VPF, but binding was restored by the addition of soluble heparin, indicating that the binding of VPF to its receptors is dependent on the presence of heparin, or on heparin-like molecules on the cell surface (124).

The binding of aFGF and bFGF on high-affinity FGF receptors is also dependent on heparin, and heparin is thought to potentiate this binding either by influencing the conformation of the FGF molecules, or by influencing the conformation of FGF receptors (125). The necessity of heparin-like molecules for the binding of VPF to its receptors was described for VPF_{165} (124), having significant affinity for heparin. It was, however, not tested if the binding of VPF_{121}, lacking the affinity for heparin, to VPF receptors is also dependent on heparin. Testing the heparin dependence for receptor binding of both VPF variants might provide interesting information about the mechanism by which heparin exerts its potentiating effect.

More recently, two receptors for VPF have been cloned. The product of the *fms*-like tyrosine kinase gene (*flt*), isolated from a human placenta cDNA library (126), was characterized as a receptor for VPF (127), forming a 205 kD complex with VPF, and

having a high affinity for it (50% inhibition of binding caused by 20 pM of unlabelled VPF; IC_{50}=20 pM). The flt receptor might be the high affinity binding site on endothelial cells reported in earlier studies. Very recently it was demonstrated that also PlGF, a growth factor related to VPF, binds with high affinity to the flt receptor (128). Another putative tyrosine kinase gene: fetal liver kinase 1 (*flk-1*) isolated from a mouse cDNA library (129) was also found to code for a VPF receptor (130). The human homologue of the *flk-1* gene was isolated independently from an endothelial cell cDNA library, and designated kinase insert domain-containing receptor (*KDR*) (131,132). Complexes of VPF with the flk-1/KDR receptor had a molecular mass of 220 kD (133) or 235 and 195 kD (132), while dissociation constants varied between 75-600 pM in different studies (130,132,133), which is in the range of the lower affinity VPF binding sites on endothelial cells found in other studies.

The sequences of the cDNAs encoding the VPF receptors flt and flk-1/KDR predict two homologous transmembrane proteins, which both have 7 extracellular immunoglobulin-like (IgG-like) domains and a tyrosine kinase domain interrupted by a so-called kinase insert. The tyrosine kinase domain is shared between very many growth factor receptors, and IgG-like domains and a kinase insert sequence are also found in e.g. the PDGF-receptor family and the FGF-receptor family (134). The VPF receptors thus have many features in common with other growth factor receptors.

Following binding of VPF to the flk-1/KDR receptor, autophosphorylation of a single protein was indeed demonstrated (130,133). An increase in intracellular Ca^{2+} was evident after binding of VPF to both the flt and the flk-1/KDR receptor (127,130), which is consistent with the finding that VPF induces rapid inositol triphosphate (IP3) formation and an increase of intracellular Ca^{2+} levels in human endothelial cells (105). Therefore, signalling of activated VPF receptors probably proceeds through activation of phosphoinositide-specific phospholipase C-γ (PLC-γ), but probably also by other pathways. It has been shown for many receptor tyrosine kinases that after autophosphorylation, intracellular messenger molecules like PLC-γ, GTPase-activating protein (GAP), and phosphatidylinositol 3-kinase (PI3-kinase) are activated, presumably through binding to phosphorylated tyrosine residues in the intracellular domain of the receptor (134). The first data on the signalling by VPF-receptors suggest that this family of receptors is not exceptional in this context.

Expression of VPF receptors is often said to be restricted to endothelial cells. This conclusion is based on *in situ* hybridizations with an *flt* probe on embryonic and adult mouse tissues (135), on hybridizations with an *flk-1* probe on developing mouse organs (133), and on the finding that transcripts of the *flt* and the *KDR* gene were present in cultured endothelial cells but absent in some non-endothelial cells (103). However, *flk-1* transcripts have been identified in murine umbilical vein stroma, which is non-endothelial tissue (130), the *flk-1* cDNA was cloned from a mouse cell population enriched in haemopoietic stem cells (129), and VPF binding sites (with a dissociation constant of 300-500 pM) have been identified on monocytes, which were also responsive to VPF (136,31). High-affinity binding sites for VPF have even been found on human melanoma

cells, HeLa cells, mouse NIH-3T3 fibroblasts, and bovine granulosa cells (137,124), but no activation of these putative receptors by VPF, nor a response of these cells to VPF, has been demonstrated.

Another resemblance with some other growth factor receptors (FGF-receptors flg and bek, 125), is that a soluble VPF receptor can be generated by alternative splicing of the *flt* pre-mRNA (138). A sequence encoding a truncated receptor was isolated from an endothelial cell cDNA library. The encoded protein lacked the membrane-spanning domain and the intracellular tyrosine kinase domains, and it was secreted from the producing cells. This soluble receptor molecule bound VPF in solution and prevented VPF to bind to cellular receptors, thus neutralizing VPF activity. Expression of this receptor variant by endothelial cells might provide a means of modulating VPF activity *in vivo*, and perhaps might be part of a feedback mechanism.

1.4 Expression of VPF in normal tissues and in pathological states

VPF was originally isolated from guinea pig line 10 tumour cells, and its activity was immediately linked to the accumulation of ascites associated with this malignancy (98). Also VEGF activity was isolated from a variety of transformed cells such as mouse tumour cell line AtT20, rat glioma cells, the human leukemia cell line HL60, and the human histiocytic lymphoma cell line U937 (51,100,101,139). VEGF was also isolated, however, from an untransformed cell type: bovine pituitary folliculo-stellate cells (140), indicating that expression of VPF/VEGF is not confined to tumour cells alone. Cloning of the cDNA sequence for VPF has made it easier to study its expression in several tissues, tumours and cell lines. Nevertheless, few quantitative studies have been published as yet comparing VPF expression levels between normal tissues and tumours. This Chapter will summarize some reports on the expression of VPF under physiological conditions, in tumours, and in other diseases (for an overview, see Table 3).

VPF expression in normal tissues

Several adult organs and tissues in guinea pig and man have been found to contain VPF mRNA, particularly lung, kidney, adrenal gland, liver, stomach, heart (especially the cardiac myocytes), as well as peritoneal macrophages (141,119). In healthy organs, with generally quiescent blood vessels, VPF may play a role in maintaining the existing density of the vasculature, or in maintaining a basal level of vascular permeability necessary for transport of, for instance, nutrients.

Expression of VPF mRNA during mouse brain development was highest during the periods of active vascularization (prenatal and postnatal) and was reduced in adult brain in which angiogenesis had ceased (110). These data suggest that VPF is involved in embryonic and fetal angiogenesis. VPF gene expression was further shown to be regulated by differentiation processes: during differentiation of mouse preadipocyte cells

A

Roles of VPF expression

In normal physiology	In pathological conditions
Embryonic and fetal angiogenesis	Wound healing
Maintenance of adult vasculature	Rheumatoid arthritis
Angiogenesis during menstrual cycle	Ovarian hyperstimulation syndrome
	Retinopathies
	Tumours

B

Features of VPF expression in tumours

Expression of VPF is often higher in tumours than in normal tissues

VPF expression can be upregulated by hypoxia

VPF accumulates in tumour blood vessels

C

Roles of VPF in tumours

VPF is essential for tumour angiogenesis

VPF causes vascular hyperpermeability and fibrin matrix generation

Does VPF influence metastasis?

Table 3. A: Summary of the normal processes and the diseases in which VPF is thought to play a role; **B:** Some generally accepted phenomena concerning VPF expression in tumours; **C:** Summary of the processes in tumour biology in which VPF is thought to play a role. For details and references see the text.

to adipocyte cells and during differentiation of mouse myogenic C_2C_{12} cells to myocytes VPF messenger levels were increased (142).

Recurring angiogenesis takes place in the female reproductive organs of mammals, coinciding with the oestrous cycle. In rat corpus luteum, in murine ovarian and uterine tissues with an expanding vasculature, and in human endometrium and myometrium expression of VPF mRNA was found (143,144,113). Spatial distribution of VPF messengers changed during the course of the oestrous cycle (113), and expression was seen especially in steroid-responsive cell types (144), suggesting that VPF expression is hormonally regulated in these tissues. Consistent with this hypothesis is the finding that

endometrium-derived carcinoma cell lines increased their VPF expression in response to oestradiol (113).

Expression of VPF mRNA has also been demonstrated in cultured rat and bovine brain capillary endothelial cells, in cultured aortic smooth muscle cells, and in human brain capillary pericytes (111, 145, and Chapter 3). If these cells express VPF also *in vivo*, autocrine stimulation of endothelial cells would be possible, and endothelial cells would be stimulated permanently by directly surrounding cells. However, data obtained with cells *in vitro* should be interpreted cautiously, as cells growing in culture may behave differently from cells *in vivo*.

VPF expression under pathological conditions

After wounding of skin, a healing process takes place in which also angiogenesis is involved. During this process, keratinocytes at the wound edge, and keratinocytes migrating to cover the wound surface were shown to have markedly increased levels of VPF mRNA compared to resting keratinocytes (107). This upregulation suggests that VPF is an important factor in neovascularization during wound healing.

VPF expression is also thought to play a role in a number of diseases (see also Table 3A). VPF expression was shown to be much higher in inflamed rheumatoid arthritis (RA) compared to other forms of arthritis, like osteoarthritis. VPF production was predominantly seen in the synovial lining, especially by macrophages. High levels of VPF were also found in RA synovial fluid, an effusion rich in plasma proteins (72,146). VPF therefore might play a role in the vascularization of RA synovial tissue, which is rich in blood vessels, and also in the enhanced vascular permeability and the accumulation of synovial fluid associated with RA.

Ovarian hyperstimulation syndrome (OHSS) is an overreaction of the ovarium to ovulation induction therapy, and is characterized by effusion of protein-rich fluid from blood vessels into the peritoneal cavity. This ascites fluid was found to contain significant amounts of VPF, which may therefore be responsible for the observed vascular hyperpermeability (147).

Expression of VPF in human retina has recently been found by several authors, and is thought to play a role in the pathogenesis of eye diseases involving ocular neovascularization, like diabetic retinopathy (148-150).

VPF expression in tumours

Notwithstanding the fact that VPF is expressed, and is probably important, in many physiological situations, much of the research on VPF has focused on its role in tumour biology. For a schematic overview of the present ideas about VPF expression in tumours, and of the presumed roles of VPF in tumour biology, see also Table 3 (B and C).

It is generally assumed that VPF expression levels correlate with the tumourigenic potential of cells, and that VPF levels in tumours are higher than in surrounding normal

tissue. One of the earliest observations leading to this hypothesis was that guinea pig line 10 carcinoma cells, and a series of human tumour cells (osteogenic sarcoma, bladder carcinoma, cervical carcinoma, and fibrosarcoma cells) produced and secreted high levels of VPF activity (151). Most interestingly, two of these tumourigenic cell lines were derived from non-tumourigenic parental cell lines which secreted much less VPF activity. Later it was also found that human tumourigenic fibrosarcoma and osteosarcoma lines produced higher levels of VPF mRNA than related but non-tumourigenic fibroblast and osteosarcoma lines (141). Also, expression of VPF mRNA in transformed and poorly differentiated phaeochromocytoma cells was down-regulated during differentiation into neuron-like cells (142).

Studies with human, mostly colonic, adenocarcinomas revealed that they often contained higher levels of VPF messenger than less malignant adenomas, and than surrounding normal epithelium and mucosa (142,152). Also in most well-vascularized human renal cell carcinomas increased messenger levels of VPF, as well as of the related factor PlGF, were found compared to adjacent normal kidney tissue (117).

Many observations have led to the idea that hypoxia may be an important VPF-inducing mechanism in tumours: locally reduced oxygen pressure due to insufficient vascularization might induce VPF expression, in an attempt to stimulate further angiogenesis and thereby to improve oxygen supply. Different groups have demonstrated an upregulation of VPF transcript levels adjacent to necrotic (presumably hypoxic) areas in human and rat glioblastoma (153-155). This upregulation was likely to be caused by hypoxia, as VPF mRNA levels were also increased in glioma cell lines cultured under low oxygen tension (153,155). In human adenocarcinomas increased VPF messenger levels were also found around necrotic sites, although this was only observed in tumour stroma but not in tumour cells (152).

VPF secreted by tumour cells is believed to accumulate in nearby tumour blood vessels. In solid guinea pig line 10 tumours and in human lymphomas, VPF mRNA was found to be synthesized in tumour cells, but upon immunohistochemistry using an anti-VPF antibody staining was observed mainly on the endothelium of tumour blood vessels and of blood vessels immediately adjacent to the tumour (156). No staining for VPF was found in more distant blood vessels. Other studies have revealed that transcripts for the VPF receptors flt and flk-1/KDR are present in higher amounts in tumour endothelial cells than in endothelial cells outside of the tumours (152,154,155). This not only suggests that VPF accumulates in tumour blood vessels because many binding sites for VPF are present there, it also suggests that tumour blood vessels are more responsive to VPF than normal blood vessels.

Does VPF expression actually play a role in the development of the tumour vascular bed? There is indirect evidence that VPF does indeed influence tumour neovascularization. In glioma, the sites of highest VPF production - near necrotic foci - were surrounded by clusters of newly formed microvessels (153), thus showing a clear spatial correlation between VPF expression and angiogenesis.

Transfection experiments have recently provided direct evidence for the importance of VPF in tumour angiogenesis and tumour growth. By transfection of non-tumourigenic Chinese hamster ovary (CHO) cells with a VPF expression construct, these cells acquired the ability to proliferate in nude mice and to form vascularized lesions (157). Similarly, transfection of the human tumour cell line HeLa with a VPF expression construct conferred to these cells a growth advantage in nude mice compared to control cells, which was associated with a higher angiogenic activity in the xenografts (158). In Chapters 3 and 4 of this thesis, experiments will be described in which the role of VPF expression in tumour angiogenesis is determined, using VPF-transfected melanoma cell lines growing as tumours in nude mice.

The importance of VPF in tumour angiogenesis was also demonstrated through inhibition of VPF activity by use of neutralizing antibodies. The growth-stimulatory effect of enhanced VPF expression by a transfected HeLa cell line was suppressed by such a treatment (158). In xenografts of rhabdomyosarcoma and glioblastoma cells in nude mice treatment with a neutralizing antibody against VPF also inhibited tumour growth, and decreased the vascular density of the lesions. The antibody did not affect the growth rate of the tumour cells *in vitro* (52). In another elegant approach VPF activity was inhibited by virus-mediated gene transfer into endothelial cells *in vivo* of a sequence encoding a dominant-negative mutant of the flk-1 VPF receptor. Glioblastomas in nude mice were growth inhibited by this treatment, and ended up as a non-vascularized and highly necrotic tumour cell mass (53).

VPF expression by tumours may also enhance the permeability of tumour blood vessels. This was demonstrated by the fact that the relative expression levels of VPF in a series of tumour cell lines correlated with the *in vivo* vessel permeability in tumours developing from these lines after injection into nude mice (159). Furthermore, the accumulation of VPF on the endothelium of tumour blood vessels was associated with hyperpermeability of these vessels for macromolecules (156). A VPF-induced enhancement of vascular permeability may facilitate tumour vascularization, as vessel permeability can lead to the deposition of a fibrin matrix around existing vessels, which in its turn is a substrate for developing tumour stroma and ingrowing capillaries (reviewed in 160).

Since angiogenesis is an important step in metastasis, VPF expression might also influence this process. However, no reports have as yet been published which directly correlate VPF expression with metastasis.

VPF in tumour diagnosis and potential clinical applications

The many observations on VPF expression in tumours indicate that the level of VPF expression in suspect cancers might be an indicator for prognosis. On the other hand, VPF expression was also found in normal tissues (presumably in lower quantities), and was associated with other diseases than cancer as well, as discussed above. This can hamper the use of VPF as a prognostic marker for cancer. Nevertheless, methods

determining VPF protein or messenger levels are being developed, and may become important tools in tumour diagnosis in the future.

For the measurement of VPF mRNA levels in tumours Northern blotting techniques might be useful, but given the small amounts of material generally available from biopsies, a quantitative RT-PCR method would probably be more practical. As yet, only a few studies have compared VPF messenger levels between malignant and less malignant or normal tissues (117,141,152). These studies have, however, not answered the question if VPF mRNA quantitations are useful in tumour diagnosis or prognosis.

For the determination of VPF protein, in the lesions themselves or in body fluids, specific immuno-assays are required. A time-resolved immunofluorometric assay (IFA) for VPF was shown to be potentially useful as a diagnostic test for malignancy: the VPF levels in human ascitic tumour effusions correlated with the presence of malignant cells in the lesions (161,162). No VPF was detected with this method in plasma and urine from tumour-bearing animals or from patients with malignant cancers.

The spatial distribution of VPF expression might also be indicative for the degree of tumour malignancy. In gliomas the distribution of VPF expression was such that the highest levels of VPF mRNA were found around necrotic foci (153,154), and there was also evidence that induction of VPF expression is mediated by hypoxia in these tumours (154,155). In other tumour types VPF expression was more homogeneous (152). It would be interesting to investigate if differences in spatial expression of VPF in tumours (induced levels around necrotic sites versus homogeneous expression) in some way relate to prognosis. Theoretically, therefore, the diagnosis of tumour malignancy may also benefit from methods that determine the spatial distribution of VPF expression, such as *in situ* hybridization and immunohistochemistry.

VPF at present is thought to have an important role in tumour angiogenesis, which in its turn is important for metastasis, as discussed before. Blocking of VPF activity therefore may inhibit both tumour progression and metastasis, and might have clinical applications. Among the many agents recently tested for their anti-angiogenic and tumour-inhibiting activity (see above), VPF inhibitors might also find a place.

In an experimental model, inactivation of VPF by transfecting endothelial cells with dominant-negative VPF receptor constructs was effective in inhibiting tumour angiogenesis and tumour growth (53). Also in animal models, neutralization of VPF by anti-VPF antibodies has been shown to suppress tumour angiogenesis and tumour growth efficiently (52,158). These approaches may also be applicable clinically, provided that virus-mediated gene transfer is accepted in clinical practice, or that sufficient amounts of neutralizing anti-VPF antibodies become available.

Blocking of VPF activity may also be achieved by the use of the serum protein α_2-macroglobulin (α_2M), which binds and inactivates VPF *in vitro* (163), or by using an excess of soluble VPF receptor, which is endogenously produced by endothelial cells, and which binds and inactivates VPF in solution (138). The ability of these endogenous proteins to inhibit angiogenesis *in vivo* has not been described yet, however.

A future approach in anti-angiogenesis might be the design of VPF receptor antagonists, which should be able to bind to, and occupy VPF receptors without activating them. Also dominant-negative VPF analogues might be designed, which should dimerize with, and thereby inactivate normal VPF polypeptide chains. Attempts to produce such analogues of other growth factors, for instance of the related factor PDGF, have shown that this will be very difficult (164). Knowledge about the structure of the protein, and about the receptor-binding domains of the factor is certainly a prerequisite for success.

1.5 Regulation of VPF expression

Expression of VPF has to be tightly regulated, as different levels of VPF activity are required in various tissues, developmental stages, and physiological circumstances. Little is known about the mechanisms regulating VPF expression, however.

There is evidence that regulation of VPF gene expression in ovarium and uterus involves hormones like oestradiol (113,144), but nothing is known about the cellular mechanisms occurring after hormone stimulation. Similarly, the mechanisms by which upregulation of VPF expression takes place in keratinocytes during wound healing (107) are as yet unknown.

Hypoxia induction of VPF gene expression

Induction of VPF expression by hypoxia appears to be a direct way of improving a vasculature that is incapable in delivering sufficient nutrients and oxygen to all cells. In such a situation cellular hypoxia can elevate the expression of VPF, which can induce the formation of the necessary new blood vessels. Hypoxia-induced expression of VPF has been shown in glioma *in vivo*, and in a variety of cell lines *in vitro*, such as glioblastoma cells, hepatoma cells, and retinal pigment epithelial cells (148,153-155,165). The expression of some other genes is induced by hypoxia as well, but the mechanisms involved in this kind of induction are only beginning to be understood (reviewed in 166). Nitric oxide (NO) plays a role in hypoxia-induced expression of the PDGF-B chain gene and the endothelin-1 gene (167), but for regulation of the VPF gene an involvement of NO has not been reported yet. There is circumstantial evidence, however, that a haeme protein acts as an oxygen sensor, and is involved in hypoxia-induced upregulation of VPF and erythropoietin (EPO) gene expression (165). In glioma cells and in hepatoma cells hypoxia-induced expression of these genes was mimicked by addition of cobalt chloride ($CoCl_2$), and was inhibited by carbon monoxide (CO): cobalt and CO are known to bind strongly to haeme groups. Hypoxia also induced the levels of c-jun and junB mRNA, while $CoCl_2$ induced c-jun and c-fos mRNA levels, which code for proteins that can dimerize to form transcription factor AP-1. Putative recognition sites for AP-1 are present in the 5'-flanking region of the VPF gene (109). Furthermore, a number of sequences 5' of the VPF gene are 90% homologous to hypoxia-responsive enhancers present 3' of the

EPO gene (165, see also Fig. 2). These *cis*-acting regulatory elements may be involved in hypoxia-induced expression of the VPF gene.

Growth factor induction of VPF expression

VPF expression has also been shown to be induced by growth factors. PDGF-BB induced the level of VPF mRNA in NIH-3T3 cells rapidly and transiently (maximum level reached after 1 h, 168). In monocytic U937 cells, mouse embryo fibroblasts, and human lung adenocarcinoma cells VPF mRNA levels were elevated by exposure to TGF-ß1, with a maximum reached after 4-8 h. In the fibroblasts the VPF protein level was shown to be increased as well (169,170). Induction of VPF expression - in tumour cells and in infiltrating cells - might be one of the ways by which TGF-ß exerts its angiogenic activity *in vivo* (see above).

Growth factor induction of VPF expression is probably mediated via a protein kinase C (PKC)-dependent pathway, since the PKC-activating phorbol ester PMA increased the VPF messenger levels in U937, NIH-3T3, preadipocyte, and vascular smooth muscle cells (168,169,142,109), and PDGF-induced upregulation of VPF mRNA levels in NIH-3T3 cells was abolished by downregulation of PKC (by preincubation with PMA, 168).

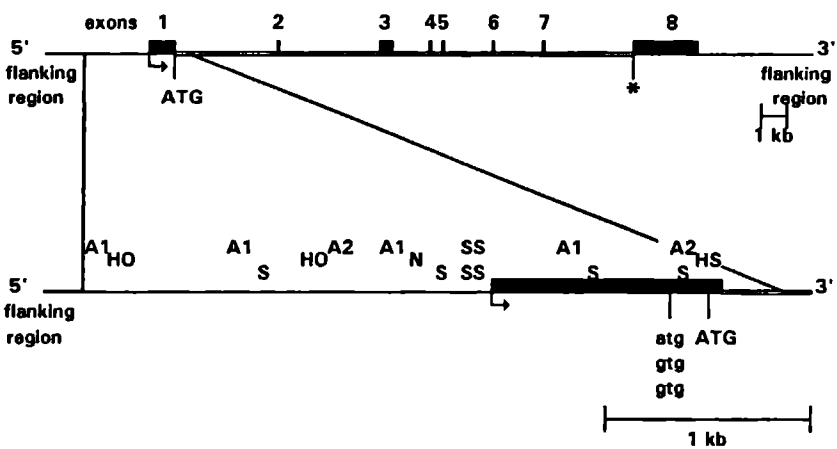

Figure 2. Schematic illustration of the VPF gene structure. The *upper line* shows the 8 exons (solid bars), the introns (double lines), and the 5' and 3' flanking regions of the gene (single lines). The transcription initiation site (→), the putative translation initiation codon (**ATG**) and the stopcodon (*) are also depicted. The *lower line* is an enlargement of a 5' flanking region of the VPF gene and the first exon. This also shows three alternative out-of-frame initiation codons (**atg** and **gtg**) and a number of sequences closely matching the consensus for transcriptional regulation elements: **A1**, AP-1 binding site; **A2**, AP-2 binding site; **N**, nuclear factor 1 binding site; **HS**, heat shock element; **HO**, hypoxia-responsive enhancer; **S**, Sp1 binding site (refs. 109,167).

VPF mRNA in mouse adipocytes was also induced by cyclic AMP analogues, which suggests that also cAMP-dependent protein kinase is involved in a pathway leading to enhanced VPF expression (142).

Transcriptional regulation

Ultimately, induction of VPF expression (by growth factors or otherwise) will probably be the result of binding of transcription factors to elements present in the VPF gene. In the 5' flanking region, and also within the VPF gene, 4 putative AP-1 binding sites, 2 putative AP-2 binding sites, and a putative nuclear factor I binding site are present. A consensus heat shock element is present in the VPF gene, which may be involved in induction of VPF gene expression in response to stress. Furthermore, 8 GC box sequences, which are putative Sp1-binding sites, are present in the regulatory region of the VPF gene, while a TATA box is absent close to the start site of transcription (109, see also Fig. 2). The presence of Sp1 binding sites and the absence of a TATA box are features often found in house-keeping genes, but it is clear that the VPF gene is inducible by external factors, as is discussed above.

In tumour cells a constitutively high expression of VPF is often found (see above). Perhaps in these cells transformation has caused aberrant expression of oncogenes or tumour suppressor genes, which may in their turn influence VPF gene expression. Elevated expression of c-jun and/or c-fos, for instance, which form the AP-1 transcription factor, might explain increased transcription of the VPF gene, but no such relation has as yet been demonstrated. However, a mutation in tumour suppressor gene *p53* frequently found in tumours does result in increased VPF mRNA levels in NIH-3T3 cells, and potentiates PMA-stimulated VPF mRNA expression (171).

Post-transcriptional regulatory mechanisms

Transcription factors obviously regulate expression at the level of transcription, but VPF expression may also be regulated post-transcriptionally. As discussed above, by alternative splicing multiple protein forms of VPF are generated which differ in their affinity for heparin-like molecules, and which are stored on the extracellular matrix of the producer cells to a different extent. Thus, there is a possibility of regulating the bioavailability of VPF by selective alternative splicing. In some organs, like kidney and heart, large amounts of the mRNA coding for a cell-bound VPF variant were found (118,119), whereas in most other cases the soluble variants were found to be predominant.

Regulation of VPF expression might also take place at the level of messenger stability. VPF mRNA has a much longer half-life in the presence of the protein synthesis inhibitor cycloheximide (165), indicating that its breakdown is dependent on active processes. Regulation of VPF mRNA stability may be possible, although it is not known by what

mechanisms the mRNA is destroyed, and by what mechanisms this breakdown may be regulated.

VPF expression may further be regulated at the translational level. In the VPF messenger, 5' of the proposed AUG translation initiation site there is one extra out-of-frame AUG codon, followed three codons further by a stop codon. In addition, there are two potential out-of-frame GUG initiation codons (109, see also Fig. 2). Furthermore, the 5' non-coding sequence immediately upstream of the initiation site is extremely GC-rich (109). These features have also been found in the messengers for other growth factors, like those encoding the PDGF-A and B chains (172). The sequences upstream of the initiation site of the PDGF-B chain messenger have a profound inhibitory effect on translation efficiency (173,174). In endothelial cells an alternative PDGF-B chain mRNA was expressed which lacks these 5' translation inhibitory sequences (175). Also in the VPF messenger the 5' non-coding region may inhibit translation, but no experiments have as yet been published confirming this.

1.6 Structural properties of the VPF protein

Properties of the VPF protein and regions essential for biological activity

VPF is formed from a preprotein containing a leader sequence which is needed for secretion of VPF, and which is cleaved off during secretion. The mature, secreted VPF polypeptide is largely N-glycosylated, presumably on the asparagine residue on position 75 in man (or residue 74 in most mammals) (51,101,114). Glycosylation is not essential for biological activity of VPF, but enhances the secretion of VPF from the producer cells (114).

As stated before, VPF exists in several molecular variants due to alternative splicing of exons 6 and 7 from its pre-mRNA (112). The VPF variants differ especially in their cellular retainment (84). The smaller VPF variants appear to be the predominant products of most cell types, and these are also the efficiently released variants. Electrophoresis under reducing conditions of medium conditioned by VPF-producing cells generally yields VPF bands between 15 and 24 kD, typical for VPF_{121} and VPF_{165}. For instance, guinea pig line 10 tumour cells mainly secreted glycosylated VPF proteins of 19.5 and 24 kD, along with some non-glycosylated VPF proteins of 15 and 20 kD (114). Under non-reducing conditions these proteins migrated as a broad band of between 34 and 43 kD (98), indicating that native VPF occurs as an S-linked dimer. The larger VPF variants of 189 and 206 amino acids are rarely seen, but if expressed they appear as bands of somewhat higher molecular mass (mainly 27 and 29 kD after reduction, 112). The different splice variants, either glycosylated or non-glycosylated, make many combinations of homodimers and heterodimers possible.

The amino acids encoded by the alternatively spliced exons 6 and 7 do not appear to be essential for biological activity, as VPF_{121}, which lacks these residues, is biologically

active in several assays (112). The 6 most C-terminal residues, encoded by exon 8, and present in all molecular variants of VPF, probably are not essential for biological activity either, since by plasmin cleavage of VPF_{189} the N-terminal part of this protein (migrating as a 17 kD band upon reduction) is released which is biologically active (112). Exon 1 only encodes residues of the signal peptide, which is responsible for secretion, and perhaps also for correct protein folding, but is no longer present in the mature protein itself. The region relevant for biological activity of VPF can thus be limited to the residues encoded by exons 2-5.

Antibodies produced against peptides corresponding with amino acids 1-26 and 171-189 of VPF_{189} (i.e. reacting with the extreme N-terminus and C-terminus) showed much better binding to native VPF, and achieved much higher inhibition of VPF activity than antibodies raised against peptides corresponding with the internal part of the VPF sequence (176). These results suggest that the N-terminus and the C-terminus are the exposed regions of the VPF protein in solution, and that these regions are essential for biological activity. These conclusions are somewhat premature, however. The extreme C-terminus was shown in other experiments to be non-essential for activity (see above). Inhibition of activity by the anti C-terminal antibody might also be caused by an indirect effect, for instance by steric hindrance of the receptor-binding site. Furthermore, some of the antibodies against the middle part of VPF reacted weakly with denatured and reduced VPF as well, indicating that their poor binding to native VPF may simply reflect properties of the antibodies, and therefore they may not give valuable information about the VPF structure.

Resemblance between VPF, PDGF, and PlGF

The preproteins of the PDGF-A and B chains, PlGF, and VPF all contain a secretory signal peptide (172,54). The mature proteins of PDGF and PlGF also resemble VPF in that they exist as S-linked dimers. The sequence identity with VPF is 15%, 18%, and 53% for the PDGF-A chain, the PDGF-B chain, and PlGF, respectively (101,54).

In the genes of all four proteins, exon 6 codes for a highly basic stretch of amino acids, that becomes part of the C-terminal half of the mature protein. Exons 6 of the genes for VPF, PDGF-A chain and PlGF are subject to alternative splicing (109,177,55). The residues encoded by exons 6 cause the proteins to remain cell-associated. In mutants of the PDGF-A and B chains lacking a signal peptide these residues have even been shown to act as a nuclear targeting signal (178,179).

The conservation of 8 cysteine residues between VPF, the PDGF-A and B chains, and PlGF (51,101,54) may be important, in that they presumably determine much of the protein structure. The role of these cysteines in maintaining the structure of PDGF is well known. The cysteines 1, 3, 5, 6, 7 and 8 form intrachain disulfide bonds which are arranged in a so-called "cystine knot", also seen in nerve growth factor (NGF) and TGF-ß2 (180,181). Cysteines 2 and 4 form disulfide bridges between two PDGF subunits in a cross-wise manner, i.e. Cys 2 is linked to Cys 4 of the other subunit, and vice versa

(182,180). Substitutions of cysteines 2 and/or 4 of PDGF by serines prevented S-linked dimerization, but such mutants of the PDGF-B chain existed as biologically active non-covalent dimers (182,183). Similar PDGF-A chain mutants were incapable of binding to PDGF receptors and did not have biological activity, however (182). There is strong evidence, therefore, that PDGF must exist as a dimer in order to bind and activate its receptor.

In Chapter 5 of this thesis, experiments will be described which test if also in VPF cysteine residues 2 and 4 are involved in intermolecular disulfide bonds, and if so, if covalent dimerization of VPF by these disulfide bridges is essential for biological activity.

Apart from the 8 cysteine residues conserved with PDGF, other cysteine residues are present in VPF and PlGF (51,101,54, see also Fig. 1), the function of which is as yet unknown. Binding of α_2-macroglobulin to VPF involves the formation of a DTT-labile disulfide bond (163), suggesting that at least one of the cysteine residues in VPF is in a reduced form.

1.7 Aims of the studies described in this thesis

The work that was carried out in the project leading to this thesis was focused on tumour-secreted factors which influence vascular endothelium, and thus tumour vasculature. Initially, attention was given to the purification, characterization, and cloning of one or more of such protein factors secreted by a human melanoma cell line. Especially factors which upregulate tissue factor expression (i.e. procoagulant activity) by endothelial cells were under survey, since such factors may induce the coagulative properties often found in tumour vasculature (25). Furthermore, such factors may cause the high sensitivity of tumour blood vessels to TNF treatment, which in some animal tumours has caused the occurrence of thrombi obstructing blood flow and haemorrhagic necrosis (35,36), and which in melanoma patients has caused significant remissions (39).

Incubation of endothelial cells in melanoma cell-conditioned medium caused the induction of procoagulant activity as well as an elevation of tissue factor messenger levels. Therefore, in the purification protocol of this melanoma-derived factor, either an assay measuring procoagulant activity (PCA), or an assay measuring tissue factor mRNA levels could be used. To achieve maximum sensitivity with the latter approach, a quantitative RT-PCR assay was set up, which made it possible to measure tissue factor mRNA levels accurately even with small amounts of endothelial cells. The development of this assay, as well as an application of this assay, are described in Chapter 2.

An article by Clauss et al. (31) showed that murine vascular permeability factor (VPF), which was until then only known to induce endothelial cell proliferation, angiogenesis, and enhanced vascular permeability, also caused increased tissue factor expression by endothelial cells. VPF was also known to be produced by a number of tumours and tumour cell lines, and therefore VPF might be (one of) the protein factor(s) that we were purifying. From the time when it was discovered that some human melanoma lines indeed expressed the mRNA for VPF, priority was given to the research on VPF.

One of the items in further study was the role of VPF in the biological behaviour of a series of human melanoma lines in nude mice. First, the question was addressed whether there was a correlation between the level of VPF expression and the metastatic potential of the melanoma xenografts. The expression of VPF was studied in these melanoma lines in cell culture as well as in the resulting tumours. The expression patterns were found to be more complicated than expected, and a clear correlation with metastatic frequency could not be found. In order to obtain more evidence for a role of VPF in melanoma angiogenesis and metastasis, the effect of altered expression levels of VPF - achieved by stable transfections of one of the melanoma lines with VPF expression constructs - on the development of the tumour vascular bed and on the metastatic potential were studied. The results of these investigations are described in Chapters 3 and 4.

Finally, studies on the structure/function relationship of the VPF protein were initiated, analogous to studies performed by others on PDGF (182). For this purpose a number of VPF mutants was generated with one of the cysteine residues changed to a serine residue. These mutants were expressed in mammalian cells which allowed the synthesis of

functionally active VPF wild type protein. The mutant proteins were tested for their ability to dimerize, and were tested for biological activity in a number of assays, including, again, the procoagulant assay (PCA). Conclusions could be drawn from these studies regarding the relation between dimerization and biological activity, and about disulfide bonds essential for VPF dimerization. These experiments are presented in Chapter 5.

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Chapter 2

Measurement of Tissue Factor Messenger RNA Levels in Human Endothelial Cells by a Quantitative RT-PCR Assay

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We have developed a sensitive and quantitative RT-PCR assay for the determination of tissue factor (TF) mRNA levels in human cells. An *in vitro* synthesized internal standard RNA was used to correct for differences in reverse transcription or amplification of various RNA samples. The PCR products were quantitated by hybridization. The sensitivity was such that less than 0.2 μg of total endothelial RNA sufficed to measure its TF mRNA content. The RT-PCR assay was used to determine TF mRNA levels in endothelial cells treated with a factor from human melanoma cells and/or TNF. In this way the amount of TF mRNA could be induced to a level that was at least 80-fold higher than that in non-induced cells. This increase was in the same order of magnitude as the induction of measured TF activity.

Introduction

Expression of procoagulant activity by the endothelium is a feature that is usually associated with pathological phenomena. For this reason it is important to study the mechanisms underlying the activated procoagulant state. Several agents such as LPS, PMA, IL-1 and TNF (1-4) as well as some tumour-derived factors (5-8) are known to act as inducers of procoagulant activity. Although some of these factors can act in a synergistic fashion (6,7), it appears that they have a common target as the procoagulant activity is generally mediated by tissue factor (TF; 2,9,10). To dissect and understand the intracellular processes leading to an enhanced TF expression it is necessary to have an assay that allows the determination of both TF activity and TF messenger RNA level.

RNA levels are usually assayed by the Northern technique. However, in this technique the amount of RNA is a limiting factor. In those cases where too little RNA is available, or where specific mRNA levels are too low, the polymerase chain reaction preceded by reverse transcription (RT-PCR) is the obvious way to improve the sensitivity of detection. To compare levels of mRNA quantitatively by RT-PCR, some precautions must be taken and internal controls must be included in the assay - for examples see (11-15). In this paper we present a quantitative RT-PCR assay for TF mRNA. As an internal standard we used an *in vitro* produced control RNA which is amplified by use of the same primers as TF mRNA and which yields a PCR product of a greater length than the TF messenger product. In an application of this technique, we have measured the non-induced level of TF mRNA in cultured human endothelial cells, and we followed the changes in the levels of TF mRNA in these cells after stimulating procoagulant activity by TNF and/or by a protein factor derived from a human melanoma cell line. TF mRNA levels were elevated to about 80-fold the basal level, which approximately equaled the increase in procoagulant activity.

Materials and methods

Chemicals and materials

Normal tissue cultureware was from Costar (Cambridge, MA, USA) and fibronectin coated tissue cultureware was from Collaborative Biomedical Products (Bedford, MA, USA). EMEM and L-glutamine were from Flow laboratories (Irvine, UK); FCS from Integro (Zaandam, The Netherlands) and NCS from Gibco BRL (Paisley, UK). Gentamycin was from the Schering Corporation (Amstelveen, The Netherlands); heparin from Organon Teknika (Boxtel, The Netherlands); trypsin from Difco (Detroit, MI, USA) and polymyxin B was from Pfizer (Rotterdam, The Netherlands). Ribonucleotides, deoxyribonucleotides and random hexamer oligonucleotides were from Pharmacia LKB (Woerden, The Netherlands). Calcium-Thromboplastin, Collagenase, Klenow enzyme, T7-RNA polymerase and the cDNA synthesis kit were from Boehringer (Mannheim,

Germany) and recombinant human TNFα was a kind gift of Boehringer (Ingelheim, Germany). RQ1-DNase was from Promega (Madison, WI, USA); AMV-reverse transcriptase was from Stratagene (San Diego, CA, USA) and Supertaq polymerase was from HT Biotechnology (Cambridge, UK). [α-^{32}P]dATP (3000 Ci/mmol) was from Amersham (Amersham, UK). All other chemicals were reagent grade and purchased from standard suppliers. Oligonucleotide primers were synthesized by a Millipore 8400 Cyclone plus nucleic acid synthesizer (Bedford, Ma, USA) and PCR reactions were performed in a Bioexcellence PHC-1 DNA incubator. Autoradiographs were scanned with an Ultroscan XL Enhanced Laser Densitometer from LKB (Bromma, Sweden).

Cell culture

The human melanoma cell line BLM was cultured as described elsewhere (16). Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment essentially as in (17). They were cultured in Eagle's modified essential medium (EMEM) supplemented with 15% heat inactivated human serum, 15% FCS, 2 mM L-glutamine, 150 μg/ml endothelial cell growth factor (isolated from calf brain as described in (18)), 10 mM HEPES pH 7.3, 5 U/ml heparin and 40 μg/ml gentamycin in fibronectin-coated tissue culture flasks. Cells were detached from their flasks using 0.125% trypsin, 0.2% EDTA in PBS, and split 1:2 or 1:4 every 2 to 3 days.

Procoagulant assay

Procoagulant activity was determined in a clotting assay essentially as in (19). Endothelial cells were grown in 30 mm wells to confluence. Medium was replaced by PMB medium (EMEM supplemented with 15% newborn calf serum, 50 μg/ml polymyxin B, 2 mM L-glutamine and 40 μg/ml gentamycin). After the addition of procoagulant inducing agents, cells were incubated for 5 hours and then suspended by scraping with a rubber policeman. Cells were washed in 0.5 ml veronal buffer, and finally resuspended in 100 μl veronal buffer (7 mM veronal, 4 mM Na-acetate, 130 mM NaCl, pH 7.4). After the addition of 100 μl 20 mM $CaCl_2$ and 100 μl human plasma, the time needed for fibrin clot formation at 37°C was recorded. All determinations were performed in duplicate. Values were converted to TF units, using a standard curve prepared by diluting Calcium-Thromboplastin in clotting buffer. One unit of TF activity was assigned arbitrarily as the equivalent of a 10,000-fold dilution of the stock concentration prescribed by the manufacturer.

Isolation of RNA

Cytoplasmic RNA was isolated from HUVEC by the method described in (20). RNA concentration was measured spectrophotometrically at 260 nm (21).

Analysis of RNA by Northern hybridization

Samples of RNA were denatured in formaldehyde buffer containing 50% formamide (formaldehyde buffer is: 6.5% formaldehyde, 25 mM NaH_2PO_4/Na_2HPO_4, pH 7.0) at 55°C for 15 min and run on a 1% agarose gel in formaldehyde buffer. RNA was then transferred to nitrocellulose in 20 x SSC (1 x SSC is: 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0) and baked at 80°C for 2 h under vacuum (21). Hybridizations were performed overnight at 45°C in 50% formamide, 6 x SSC, 0.1% SDS, 0.1 mg/ml denatured herring sperm DNA and 5 x Denhardt's (21) and filters were subsequently washed 3 x 20 min in 0.2 x SSC, 0.1% SDS at 65°C and exposed to X-ray films at -70°C. The following probes were used: the 1.03 kb insert from HTF8, a cDNA clone comprising the complete human tissue factor protein coding region (22), and the insert from pHGR21, a cDNA clone encoding human poly-ubiquitin (23). The fragments were labelled with [α-^{32}P]dATP after random hexamer priming (24).

Control RNA for the RT-PCR

The insert from HTF8 (22) was recloned into the SmaI site of pUC19, yielding the plasmid pTF1. A 0.4 kb *Eco*R1 fragment containing a non-mammalian sequence -ay1, a repeat unit from the Drosophila hydei Y-chromosomal gene "nooses" (25)- was ligated into the *Eco*R1-site at the end of the 5th exon (26; position 817) (Fig. 1) by standard techniques (21). The insert of the resulting clone pTFay1 was transferred to pGEM-9Zf-, yielding clone pGEM-TFay1. Control RNA was synthesized *in vitro* using 250 ng of *Hin*dIII-digested pGEM-TFay1 DNA in 40 mM Tris/HCl pH 8.0, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 1 mM of each NTP, 100 U RNasin, and 100 U T7 RNA polymerase in a 50 μl volume (21). The reaction was performed at 37°C for 2 h. Plasmid DNA was then digested by treatment with RQ1-DNaseI. RNA was extracted with phenol, ethanol precipitated and redissolved in distilled water. To confirm the integrity and correct length (1.5 kb) of the control RNA, and to determine its concentration, a sample of this RNA was run on a 1% agarose/formaldehyde gel (as described above) together with a dilution range of *Hin*dIII-linearized and denatured pTF1 plasmid DNA. A nitrocellulose blot of this gel was hybridized to the TF probe at 44°C. No residual pGEM-TFay1 plasmid DNA could be detected in the RNA preparation.

RT-PCR protocol

Preparations of HUVEC RNA and control RNA were diluted in distilled water containing 100 ng/μl carrier (yeast ribosomal) RNA. HUVEC RNA (between 10 and 900 ng) and control RNA (between 0.1 and 1.0 pg) (Fig. 1) were mixed and 2 μl of 5 x cDNA first strand buffer (0.25 M Tris/HCl pH 8.3, 0.25 M KCl, 50 mM $MgCl_2$, 50 mM DTT), 2.5 μl of dNTP solution (2 mM each), 1 μl (0.5 μg) of the TF specific reverse primer (5'-CAGTGCAATATAGCATTTGCAGTAGC-3') (Fig. 1) and distilled water up

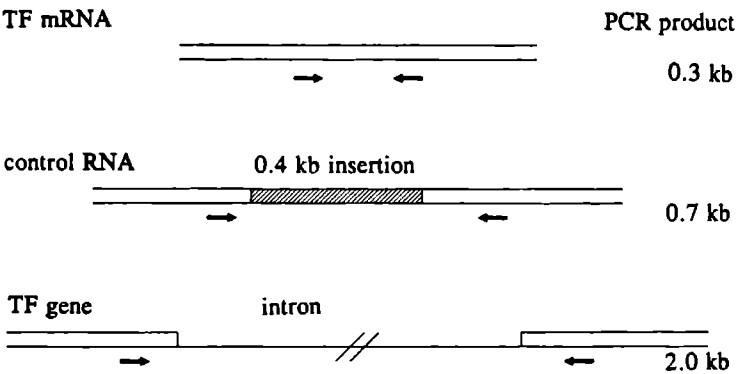

Figure 1. Outline of the RT-PCR assay. Specific primers corresponding to positions 723-748 (forward) and 1005-980 (reverse) (22) amplify a 0.3 kb region of the tissue factor mRNA consisting of parts of the 5th and 6th exon. *In vitro* transcribed control RNA added to each reaction has a 0.4 kb insertion and gives rise to a 0.7 kb PCR product. Contaminating genomic DNA should yield a 2.0 kb fragment which spans the 1.7 kb 5th intron (26).

to 9.5 μl were added. The mixture was incubated at 65°C for 5 min and directly placed on ice. Then 0.25 μl (6 U) of RNasin and 0.25 μl (6.5 U) of AMV reverse transcriptase were added. The reverse transcriptase reaction was incubated for 1 h at 42°C. Subsequently the nucleic acids were ethanol precipitated and redissolved in 20 μl distilled water. PCRs were performed with one fifth or one half of the cDNA, to which 5 μl of 10 x Taq polymerase buffer (100 mM Tris/HCl pH 9.0, 500 mM KCl, 0.1% gelatin, 1% Triton X-100), 5 μl of dNTP solution (2 mM each), 1 μl (0.5 μg) of reverse primer, 1 μl (0.5 μg) of forward primer (5'-CTACTGTTTCAGTGTTCAAGCAGTGA-3') (Fig. 1), distilled water up to 50 μl, and 0.1 μl (0.5 U) of Supertaq polymerase were added. Each cycle consisted of a denaturation step at 96°C for 1 min, an annealing step at 62°C for 1.5 min, and an elongation step at 72°C for 4 min. 20 cycles were used when 1 pg of control RNA was reverse transcribed; 25 cycles were used when 0.1 pg of control RNA was reverse transcribed. 10 μl and 1 μl samples of the PCR reactions were run on a 1.3% agarose gel in 0.5 x TBE (1 x TBE is 100 mM Tris, 100 mM boric acid, 2.5 mM EDTA, pH 8.2). DNA was "Southern blotted" (21) and hybridized to the TF probe at 42°C in 50% formamide, 6 x SSC, 0.1% SDS, 0.1 mg/ml denatured herring sperm DNA and 5 x Denhardt's, washed 3 x 20 min in 0.2 x SSC, 0.1% SDS at 65°C and exposed to X-ray film at -70°C. Intensities of the hybridization signals were quantified by scanning and integration of autoradiographic bands on several exposures with a laser densitometer.

Results

Concentration-dependent induction of procoagulant activity by melanoma-derived HS-1 activity

Culture supernatants of the human melanoma cell line BLM (16) were found to induce procoagulant activity on human umbilical vein endothelial cells (HUVEC). A partially purified component from this supernatant, denoted here as heparin-sepharose fraction 1 (HS-1; see legend to Fig. 2), was used in the studies reported below. HS-1 caused a dose-dependent induction of procoagulant activity that approached a maximum of about 100-fold the initial level at 100 μl HS-1. To determine the mode of cooperation between the HS-1 activity and TNF, the experiment was repeated in the presence of 50 pM TNF. Addition of TNF did cause an enhanced procoagulant activity in the presence of HS-1. However, the increase in activity was not synergistic but probably due to an additive effect of HS-1 and TNF. The maximum level of procoagulant activity was not significantly increased by addition of TNF (Fig. 2).

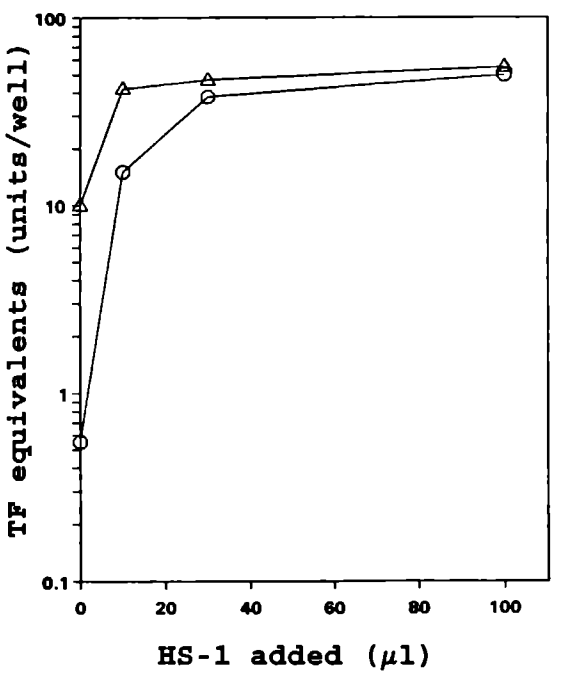

Figure 2. Procoagulant activity on endothelial cells treated with HS-1 in the presence or absence of TNF. Serum-free BLM culture supernatant was subjected to heparin-sepharose affinity chromatography. The major activity, which eluted around 0.1 M NaCl, was called HS-1. Different amounts of HS-1 were assayed alone (○) or together with 50 pM TNF (△). Clotting times were compared to a standard curve of TF activity prepared using serial dilutions of Ca-Thromboplastin. 1 unit was arbitrarily defined as the activity matching to a 10,000-fold dilution of Ca-Thromboplastin stock solution. Values are the average of duplicate measurements. Standard deviations were omitted since they were small compared to the size of the symbols in the figure (3-7% of the values).

HS-1 also increases the TF mRNA contents in HUVEC

To determine the effect of HS-1 and TNF on the tissue factor mRNA levels HUVEC were incubated with varying amounts of HS-1 in the absence or presence of TNF. Total RNA was isolated 2 h after the start of the incubations - the time point when maximal TF mRNA levels were found upon treatment with LPS and PMA (27) - and 2 μg aliquots were Northern blotted (Fig. 3). In non-treated HUVEC TF mRNA could hardly be detected, whereas in HS-1 and TNF-treated HUVEC two TF mRNA bands of 2.3 kb and 1.9 kb became apparent after prolonged exposure. The appearance of a major band of 2.3 kb is in accordance with the data from others, while double bands were also found earlier (6,27). These results showed a clear induction of the TF mRNA upon HS-1 treatment. However, the intensities of the signals were just above the limit of detection, which makes quantitation of these results highly unreliable.

To improve the sensitivity of TF mRNA determination, we developed a quantitative RT-PCR assay. The strategy is outlined in Fig. 1: primer sites on the TF transcript were chosen such that they are located in different, adjacent exons, thus allowing a distinction between amplification of TF mRNA and of possible genomic DNA contaminants. In addition, an RNA to be used as an internal control was synthesized. This RNA has the same primer sites but should yield a PCR product 400 nucleotides longer than the one derived from authentic TF mRNA.

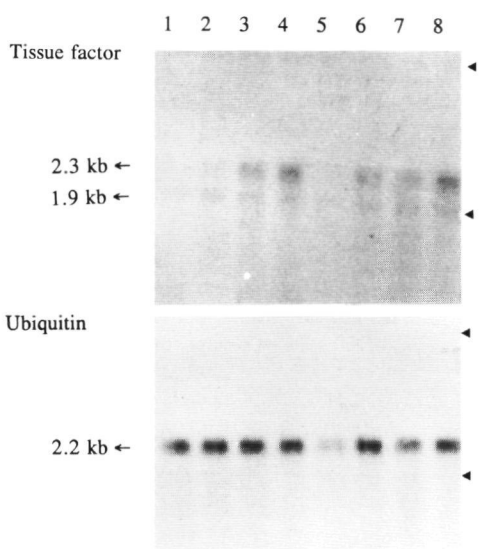

Figure 3. Northern analysis of tissue factor mRNA levels. HUVEC were incubated with: 0 μl (**1**), 10 μl (**2**), 30 μl (**3**), 100 μl (**4**) of HS-1 alone, and with: 0 μl (**5**), 10 μl (**6**), 30 μl (**7**), 100 μl (**8**) of HS-1 along with 50 pM TNF. Samples of total RNA (approximately 2 μg except for Lane **5**) isolated from these cells were loaded on a gel. The resulting Northern blot was first hybridized to a tissue factor probe (upper part) and then hybridized to a ubiquitin probe (lower part). Autoradiographs were exposed for 14 days with two intensifying screens (tissue factor) and for 2 days with one intensifying screen (ubiquitin). Arrowheads indicate postitions of 18S and 28S ribosomal RNA. Note that much less RNA was loaded in lane **5**.

Challenging the RT-PCR

First, the question was addressed whether under the conditions applied the RT-PCR yields quantitative results. To test this RNA was isolated from HUVEC which had been incubated with 100 µl of crude BLM culture supernatant for 2 hours. This amount of crude supernatant is equivalent in activity to approximately 30 µl of partially purified HS-1 (see Fig. 2) and these cells should contain enhanced levels of TF mRNA (see Fig. 3). Aliquots of this RNA (from 10 to 900 ng) were mixed with known amounts of control RNA (0.1 or 1.0 pg) and subjected to the RT-PCR. To visualize and quantify the TF specific PCR bands, the PCR products were Southern blotted and hybridized with a TF probe. As expected, higher inputs of HUVEC RNA yielded more 0.3 kb PCR product relative to the 0.7 kb control product (Fig. 4). The output ratio TF mRNA product : control product was quantified by densitometry and plotted as a function of the amount of input RNA (Fig. 5). Curves were drawn from the series using 0.1 pg of control RNA (A and C), and 1.0 pg of control RNA (B and D). Theoretically, these are expected to be

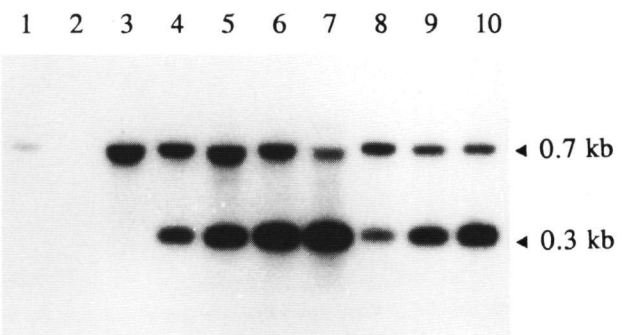

Figure 4. RT-PCR with different amounts of induced HUVEC RNA and control RNA. RNA was isolated from HUVEC which were incubated with 100 µl of crude BLM culture supernatant. It was mixed with *in vitro* transcribed control RNA and reverse transcribed. One fifth of the cDNA was used for PCR. 10 µl samples of the PCR products were separated on gel, blotted and hybridized to a tissue factor probe. The autoradiograph was exposed for 3 days without an intensifying screen. **1:** 1 pg control RNA - no reverse transcriptase was used in the first reaction; **2:** yeast ribosomal RNA (carrier); **3:** 0.1 pg control RNA; **4:** 0.1 pg control RNA and 10 ng HUVEC RNA; **5:** 0.1 pg control RNA and 30 ng HUVEC RNA; **6:** 0.1 pg control RNA and 100 ng HUVEC RNA; **7:** 0.1 pg control RNA and 300 ng HUVEC RNA; **8:** 1 pg control RNA and 100 ng HUVEC RNA; **9:** 1 pg control RNA and 300 ng HUVEC RNA; **10:** 1 pg control RNA and 900 ng HUVEC RNA. Reactions **1-7** were cycled 25 times, reactions **8-10** were cycled 20 times. Note that a faint band of 0.7 kb appeared when control RNA was subjected to the PCR without prior reverse transcription (lane **1**). This may be caused by a contamination with pGEM-TFay1 plasmid DNA. It might also be the consequence of a slight reverse transcriptase activity present in Taq polymerase (28). Its contribution to the 0.7 kb band in the normal RT-PCR reactions can be neglected. In none of the total RNA preparations were bands of 2.0 kb resulting from amplification of genomic DNA seen.

straight lines. The slopes of some curves however showed a slight decrease. This non-linearity is probably caused by saturation of the hybridization with bands that contain large amounts of PCR product (this decrease apparently is more pronounced in Figs. 5 A and B where more PCR product was loaded). Results should preferably be quantified from hybridizations on small amounts of PCR product. The amount of TF mRNA in the RNA preparation used in this experiment was estimated from three points in Figs. 5 C and D each. The mean value found was 19 pg/μg total RNA, with a standard deviation of 21% (n=6). Mean values calculated independently from Figs. 5 C and D were such that standard deviation was 18% (n=2). These experiments thus show that this RT-PCR can measure TF mRNA levels reproducibly. Within limits, the results are not affected significantly by the amount of endothelial cell RNA and control RNA used in the assay.

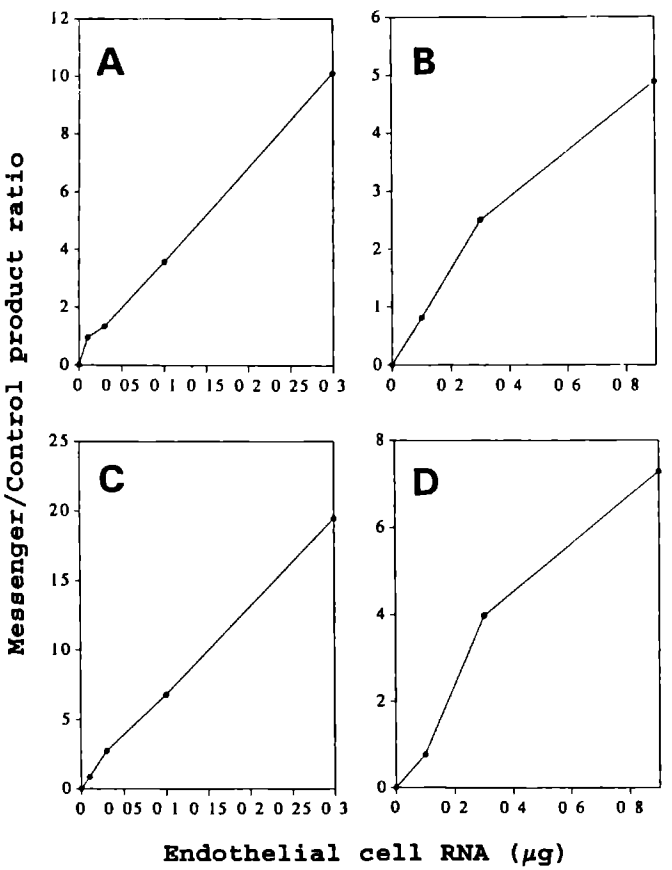

Figure 5. Quantitation of RT-PCR test results. Intensities of bands on autoradiographs (such as shown in Fig. 4) were measured by densitometry. Ratios of intensities of the 0.3 kb and the 0.7 kb bands were plotted against the input amount of HUVEC RNA. **A:** 0.1 pg control RNA; 10 μl product loaded. **B:** 1.0 pg control RNA; 10 μl product loaded. **C:** 0.1 pg control RNA; 1 μl product loaded. **D:** 1.0 pg control RNA; 1 μl product loaded.

The induction of TF mRNA by HS-1 measured by RT-PCR

The results presented in Figs. 4 and 5 show that the RT-PCR can indeed be used for a quantitative measurement of TF RNA. Hence we applied this method to quantitate the changes in TF mRNA levels in HUVEC treated with different amounts of HS-1 with and without TNF. RT-PCR was performed with 0.2 μg samples of HUVEC RNA which were mixed with 0.2 pg or 1 pg of control RNA. The PCR signals obtained are shown in Fig. 6 and show the expected trend towards an increase in TF mRNA upon stimulation with HS-1. To correct for differences in reverse transcription and amplification, the PCR mRNA signal (the 0.3 kb band) was divided by the control RNA signal (the 0.7 kb band). The ratio obtained was plotted against the added amount of HS-1 in Fig. 7. Again, the effects of TNF and HS-1 were additive rather than synergistic. Although the TF mRNA content (Fig. 7) appears to reach its plateau earlier than procoagulant activity (Fig. 2), both curves are quite comparable.

To illustrate the potential of this method, we have estimated from our data a number of TF mRNA levels in endothelial cells upon induction with TNF and/or HS-1. In RNA from non-treated endothelial cells the messenger/control product ratio (m/c) was 0.048. A TF mRNA level of 0.54 pg/μg was calculated. In cells treated with 50 pM TNF (m/c ratio of 0.67) and in maximally induced cells (m/c ratio of 3.94) TF mRNA values of 7.5 pg/μg and 44 pg/μg were found, respectively. Hence, the maximum induction of TF mRNA achieved in this experiment was about 80 times the basal level. Higher inductions might also be possible however, as from Fig. 7 it is not clear whether the maximum level of TF mRNA has been reached yet. Since endothelial cells isolated from human material are quite heterogeneous, the same experiment repeated with another batch of endothelial cells may yield different results.

Discussion

Previous studies have used Northern hybridizations to assay tissue factor mRNA (6,10,27). However, it is often difficult to obtain enough RNA to achieve reliable results with this technique. This recently led to the use of a semi-quantitative RT-PCR assay to determine differences in TF mRNA expression (8). We have presented in this paper a highly sensitive method for quantitative measurement of tissue factor mRNA levels in human cells, which should be applicable even with amounts of RNA much less than the 0.2 μg used by us. In our PCR assay we have used only one set of primers to amplify the messenger and control RNAs. As the specificity of primers is seldom absolute, this resulted in a smear of PCR products upon ethidium bromide staining. Hybridization with a TF probe was necessary to stain the TF specific fragments. The specificity of the PCR could have been improved by the use of a second set of primers, so-called "nested primers". However, such a procedure requires additional manipulations, while hybridization has the further advantage that band intensities can be simply quantitated.

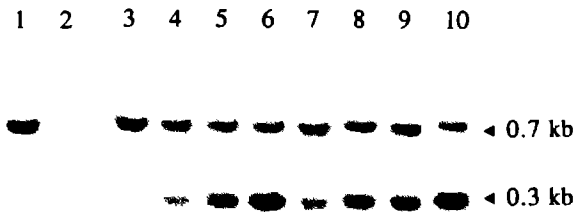

Figure 6. RT-PCR on RNA from HUVEC that were treated with various amounts of HS-1 in the presence or absence of TNF. Endothelial cells were incubated with: no HS-1 (**2,3**), 10 μl HS-1 (**4**), 30 μl HS-1 (**5**), 100 μl HS-1 (**6**) alone, and with: no HS-1 (**7**), 10 μl HS-1 (**8**), 30 μl HS-1 (**9**), 100 μl HS-1 (**10**) along with 50 pM TNF. RNA was isolated from these cells and used in the following reactions: **1:** 0.2 pg control RNA only; **2:** 0.2 pg control RNA and 0.2 μg non-induced HUVEC RNA. **3-10**: 1 pg control RNA and 0.2 μg HUVEC RNA. After reverse transcription one half of the cDNA was used for PCR. Reactions **1** and **2** were cycled 23 times, reactions **3-10** were cycled 20 times. 10 μl samples of the PCR products were separated on a gel, blotted and hybridized with a tissue factor probe. The autoradiograph was exposed for 24 h without an intensifying screen.

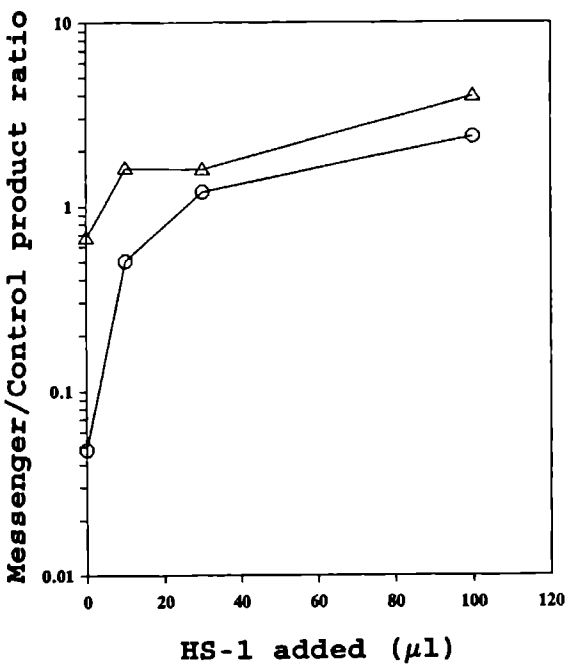

Figure 7. Quantitation of RT-PCR results. Bands as in Fig. 6, lanes **3-10**, but from blots with 1 μl of the PCR products, were quantified by densitometry. Ratios of intensities of the 0.3 kb and the 0.7 kb bands were plotted against the amount of HS-1 added to the endothelial cells. One series was performed without TNF (○), and another series was performed in the presence of 50 pM TNF (△).

The output ratio of target and control PCR fragments increased, as expected, with the input ratios of TF mRNA and control RNA. RT-PCR on dilution series of endothelial cell RNA with either 0.1 pg or 1.0 pg control RNA resulted in approximately equal estimates of the TF mRNA level, showing that this assay is applicable to a broad range of input RNA concentrations. Quantitation of the bands showed that blots are easily overloaded, causing non-linear relations between input and output ratios. Loading small amounts of PCR product should minimize this problem.

We have applied this technique to follow changes in the TF mRNA level in endothelial cells upon incubation with a factor secreted by human melanoma cells. An increase in procoagulant activity was coincident with a rise in TF mRNA levels. Upon stimulation with 100 μl HS-1 and TNF, TF mRNA levels and procoagulant activities were elevated by the same order of magnitude (80/100-fold the basal level). This correlation is not unexpected, since TF mRNA accumulation may preceed elevated TF protein expression. However, the TF mRNA level is not necessarily the only factor determining the final procoagulant activity. Post-transcriptional processes may also play an important role. Furthermore, not all TF protein that is synthesized by the endothelial cells will be assayed. A large part of the TF protein is located extracellularly, and some of this is left behind after scraping of the cells, as was shown by procoagulant assays on intact cell layers and in wells from which the cells were removed by scraping. Procoagulant activities were much higher when scraped cells were used (data not shown), suggesting that intracellular TF is released by injury of cells, or that the assay is influenced by damaged membranes. Thus, the amount of TF activity measured differs with the method used to harvest the cells. Therefore we have only compared relative procoagulant activities, and expressed them in arbitrary units.

The procoagulant inducing activity from the human melanoma cell line BLM is mediated by a protein factor, the nature of which is still under investigation. Some tumour-derived procoagulant inducing proteins isolated by others appeared to act synergistically with TNF (6,7). We did not observe this synergism between the melanoma-derived factor HS-1 and TNF, neither at the level of TF mRNA, nor at the level of procoagulant activity. This effect might nevertheless be demonstrable at lower concentrations of either TNF or HS-1.

Acknowledgements

We thank Boehringer Ingelheim for their generous gift of TNFα, and Dr. Evan Sadler for providing the cDNA probe for tissue factor.

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Chapter 3

Vascular Permeability Factor Expression Influences Tumour Angiogenesis in Human Melanoma Lines Xenografted to Nude Mice

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We studied the expression of the angiogenic factor vascular permeability factor (VPF, also called vascular endothelial growth factor/VEGF) in human melanoma cells *in vitro* and *in vivo*. Melanoma lines that develop tumours with a low metastatic potential in nude mice were found to have low expression levels of VPF *in vitro*, and the VPF expression levels in melanoma lines that yield highly metastatic xenografts were high. However, *in vivo* the correlation between VPF mRNA levels and the frequency of metastasis was lost: in all xenografts equally high levels of VPF mRNA were found, independent of the parental cell line. Hence, *in vivo* VPF gene expression was upregulated in the low expressing lines. The external factor responsible for this induction may be hypoxia, since we found that low oxygen tension caused a (reversible) increase in the VPF mRNA levels in otherwise low expressing melanoma lines *in vitro*. A melanoma line with an inducible VPF expression was engineered into a line with a constitutive VPF expression. In the xenografts from this line a change in the vascular architecture was seen, indicating that the pattern or the level of VPF expression is important for tumour angiogenesis in melanoma xenografts.

Introduction

Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), is a glycosylated, secreted protein factor that increases blood vessel permeability, stimulates endothelial cell division *in vitro*, and induces angiogenesis *in vivo* (1,2). Two different receptors for VPF have been characterized: fms-like tyrosine kinase (flt) and fetal liver kinase 1/kinase insert domain-containing receptor (flk-1/KDR). These appear to be expressed almost specifically by endothelial cells and haemopoietic cells (3-6). VPF is an important angiogenic factor, along with other factors such as acidic and basic fibroblast growth factor (aFGF, bFGF) and transforming growth factor ß (TGF-ß) (7,8). Apart from its expression by some normal, well-vascularized tissues, embryonic tissues and during wound repair (9-12), VPF has been found in many tumours and tumour cells (13). In glioblastoma VPF expression was found to be highest near necrotic areas, and clusters of newly formed capillaries were found around the sites of VPF production (14,15), suggesting that VPF can be recruited to augment angiogenesis if the tumour vasculature and therefore the oxygen supply is insufficient. The role of VPF in tumour angiogenesis was confirmed by blocking its activity in tumours by a monoclonal antibody, and by application of a dominant-negative VPF receptor mutant (16,17). These treatments led to a decrease in angiogenesis and to slower tumour growth.

Several protein variants of VPF exist due to alternative splicing of the VPF mRNA. The molecular variants differ in their efficiency of secretion. The smaller forms (VPF_{121} and VPF_{165}) are efficiently secreted by the producing cells, and can easily reach their target cells. The larger forms (VPF_{189} and VPF_{206}) are retained at the extracellular matrix, but biologically active parts of these proteins can be released by plasmin (18-20). Theoretically, different cell types may benefit from particular VPF variants. Tissue-specific expression of certain VPF messenger variants has indeed been demonstrated (19,21).

As a consequence of its proposed role in tumour angiogenesis, VPF may also facilitate metastasis, as this process is dependent on the vascular bed (22). To study the relation between VPF expression and metastasis, a panel of human melanoma cell lines was used. Xenografts of these melanoma lines in nude mice give rise to tumours with distinctly different biological behaviour. Some of these lines develop into rapidly metastasizing tumours, whereas other lines metastasize at a low frequency or very slowly (23, JR Westphal et al; manuscript in preparation). The metastatic phenotype of these lines was found to correlate with the expression of a series of genes or antigens, such as urokinase-plasminogen activator (uPA) and its type 1 inhibitor (PAI-1), various integrins, the epidermal growth factor receptor (EGF-R), thymosin ß10 and calcyclin (24-28). We report here that low and highly metastatic melanoma lines have distinctly different expression patterns of VPF. Highly metastatic melanoma lines have constitutively high levels of VPF expression, whereas the less metastatic lines have a low level of VPF expression in culture, which is elevated in mouse xenografts. *In vitro* data suggest that hypoxia may be an important trigger in this upregulation of VPF gene expression.

Transfection experiments show that an alteration of VPF expression in melanoma xenografts profoundly affects vascular architecture. Hence, at least in this melanoma model, the VPF expression pattern is an important determinant of angiogenesis, and possibly of angiogenesis-dependent biological behaviour.

Materials and Methods

Cell culture

Human melanoma cell lines were cultured as previously described (23). Transfected melanoma lines were cultured in medium supplemented with 200 μg/ml Hygromycin B (Boehringer Mannheim, Germany). Human brain capillary pericytes were isolated and cultured as described elsewhere (29). U937 cells and Balb-3T3 cells were from ATCC (Rockville, MD), and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Flow Laboratories, Irvine, UK) with 10% fetal calf serum (FCS, Gibco BRL, Paisley, UK), 2 mM L-glutamine and 40 μg/ml gentamycin (Schering Corporation, Amstelveen, The Netherlands).

Melanoma xenografts

Human melanoma cells were trypsinized and $2x10^6$ cells were injected s.c. into BALB/c *nu/nu* mice, as previously described (23). Xenografts were dissected at different time points after inoculation, measured, rapidly frozen in liquid nitrogen, and stored at -70°C.

Isolation of RNA

RNA was isolated from cultured cells either using LiCl/urea (30), or using guanidinium chloride (31). RNA from tumours and normal murine tissues was isolated after disruption in guanidinium isothiocyanate by CsCl centrifugation (32). RNA concentration was determined spectrophotometrically at 260 nm (32).

Northern blotting and probes used

Samples of total RNA were denatured in 50% formamide in formaldehyde/phosphate buffer (65 mg/ml formaldehyde, 25 mM NaH_2PO_4/Na_2HPO_4, pH 7.0) for 15 min at 55°C, run on a 1% agarose gel in formaldehyde/phosphate buffer and blotted to nitrocellulose membranes (32). As a probe for VPF mRNA, a fragment was used which comprised the protein coding region of VPF_{165}. To isolate this fragment, RT-PCR was performed on RNA from PMA-treated U937 cells (1), using reverse primer (5'-TTCCT-CCTGCCCGGCTCACCG-3') and forward primer (5'-CCCGGTCGGGCCTCCGAAAC-CA-3'). Blunt-ended PCR products were cloned into *Sma*I-digested pUC19 vector DNA

and characterized by sequencing. PCR products from the alternatively spliced messengers for VPF_{121}, VPF_{165}, and VPF_{189} were identified. A 0.6 kb VPF_{165} insert was isolated as a *XbaI*/*KpnI*-fragment and labelled. Control hybridizations were performed with a human ubiquitin probe (33), a human ß-actin probe (34), or with a *Drosophila* ribosomal RNA probe. Fragments were labelled with [α-^{32}P]dATP after random hexamer priming (35). Northern hybridizations were performed in 50% formamide, 6 x SSC, 0.1% SDS, 0.1 mg/ml denatured herring sperm DNA and 5 x Denhardt's (32) at 45°C, followed by washing in 0.2 x SSC, 0.1% SDS at 65°C. Intensities of hybridization bands were scanned with an Ultroscan XL Enhanced Laser Densitometer from LKB (Bromma, Sweden).

Analysis of alternatively spliced VPF mRNA

2-8 μg of total RNA isolated from cell lines or melanoma xenografts was reverse transcribed using 0.5 μg reverse primer (5'-TTCCTCCTGCCCGGCTCACCG-3'), 0.4 mM deoxyribonucleotides (dNTPs; Pharmacia LKB, Woerden, The Netherlands), 5 units AMV-RT (Stratagene, San Diego, CA), 5 units RNasin (Boehringer Mannheim, Germany) and reaction buffer provided by the supplier of AMV-RT, in final volumes of 10 μl, for 1 h at 42°C. After ethanol precipitation and redissolving in 10 μl distilled water, half of the cDNA was used in PCR reactions containing 0.3 μg reverse primer (see above), 0.3 μg forward primer (5'-GCACCCATGGCAGAAGGAGGA-3'), 0.2 mM dNTPs, 0.25 units Supertaq polymerase and reaction buffer (HT Biotechnology, Cambridge, UK), in final volumes of 25 μl. 20 cycles of 1 min at 96°C, 1.5 min at 52°C and 4 min at 72°C were performed. 5 μl of every reaction mix was transferred to a new tube containing the same reagents, for one more cycle (6 min at 72°C). 10 μl samples of the products were separated on a 1.5% agarose gel, Southern blotted to nitrocellulose and probed with the VPF_{165} fragment in formamide hybridization mix at 42°C.

Determination of the species origin of VPF mRNA

1-12 μg of total RNA isolated from human cell lines, murine tissues or melanoma xenografts was reverse transcribed using 0.5 μg reverse primer (5'-TTGGTGAGGTTTG-ATCCGCAT-3'), 0.4 mM dNTPs, 5 units AMV-RT, 10 units RNasin and reaction buffer provided by the supplier of AMV-RT, in final volumes of 10 μl, for 1h at 37°C. After ethanol precipitation and redissolving in 10 μl distilled water, half of the cDNA was used in PCR reactions containing 0.3 μg reverse primer (see above), 0.3 μg forward primer (5'-CGAAACCATGAACTTTCTGCT-3'), 0.2 mM dNTPs, 0.25 units Supertaq polymerase and reaction buffer, in final volumes of 25 μl. 20 cycles of 1 min at 96°C, 1.5 min at 48°C and 2.5 min at 72°C were performed. 1 μl samples of the products were transferred to new tubes with all the PCR reagents, and subjected to one more cycle (10 min at 72°C). PCR products were then precipitated and redissolved. One third was

digested with 5 units *Sty*I - which only cleaves the human product (2,36), separated on a 1.5% agarose gel, Southern blotted, and hybridized with the VPF_{165} probe.

Analysis of secreted VPF protein

Melanoma cells were cultured for 24 h in serum-free medium, when indicated with 100 μg/ml heparin. Conditioned media were centrifuged and used for VPF analysis. To correct for differences in cellular density, cells were scraped and lysed, and the cellular protein content was determined by a standard protein assay (Bio-rad, Veenendaal, The Netherlands). Based on this protein determination, samples of conditioned media derived from equal amounts of cell material were taken, and proteins were acetone precipitated, electrophoresed on 17.5% polyacrylamide gels under reducing conditions, and electroblotted to nitrocellulose membranes. Antisera against VPF were raised in rabbits using purified VPF produced in a bacterial expression system. Antiserum was diluted 1:100 or 1:250 for detection of VPF on Western blots and the immune reactions were visualized using the chemiluminescent substrate AMPPD (Tropix, Westburg, Leusden, The Netherlands) following the manufacturer's protocol. The crude antisera had some non-specific reactivity towards some bands of higher molecular mass than VPF on Western blots. Where indicated this was diminished by affinity purification of anti-VPF antibodies using purified *E. coli*-produced VPF absorbed by nitrocellulose membranes (37).

VPF induction experiments

The response to serum, fibroblast-conditioned medium or growth factors/cytokines was tested after preculturing subconfluent melanoma cell cultures in DMEM without serum for 24 h. Medium was then replaced for 4 h or 24 h by DMEM with 10% FCS, or by DMEM (with and without FCS) conditioned by Balb-3T3 cells for 24 h. Alternatively, serum-free medium was replaced for 4 h by DMEM containing 0.1% bovine serum albumin and one of the following growth factors or cytokines: 5 ng/ml epidermal growth factor (EGF, Collaborative Research, Bedford, MA); 5 ng/ml transforming growth factor alpha (TGFα, Bachem, Bubendorf, Switzerland); 2 ng/ml transforming growth factor beta 1 (TGFß1, R&D Systems, Minneapolis, MN); 10 ng/ml platelet-derived growth factor AA (PDGF-AA, a gift from Dr. C.H. Heldin, Uppsala, Sweden); 100 ng/ml basic fibroblast growth factor (bFGF, a gift from Scios Inc., Mountain View, CA); approximately 100 ng/ml VPF_{165} (in culture supernatant of COS cells transfected with plasmid containing VPF_{165} cDNA under control of an SV40 early promoter); 50 ng/ml tumour necrosis factor alpha (TNFα); 100 units/ml interleukin 4 (IL-4); 400 units/ml interferon gamma (IFNγ) (TNFα, IL-4 and IFNγ were from Boehringer Ingelheim, Germany); 100 units/ml interleukin 1 beta (IL-1ß); 50 units/ml interleukin 2 (IL-2) (IL-1ß and IL-2 were from Genzyme, Sanbio, Uden, The Netherlands). Hypoxia experiments were performed using subconfluent melanoma cell cultures in 10 mM Hepes-buffered

DMEM with 10% FCS. Rubber-capped flasks were flushed for 1 h at room temperature with 30 volumes of nitrogen that was made oxygen-free using a BASF R 3-11 catalyst (BASF, Ludwigshafen, Germany). Oxygen-free CO_2 was added up to 5%, and the flasks were then placed back at 37°C for the indicated times. At the end of each incubation, gas samples were tested for O_2 content by gas chromatography, and the pH of the media were determined. In some cases, also the Po_2 of the media were determined (CIBA-Corning 288 Blood Gas System, Houten, The Netherlands). Control experiments were performed (in air/5% CO_2) to investigate the influence of the pH of the medium. To achieve a low final pH, cells were grown in Hepes-buffered medium for several days, and to achieve a high pH, medium was replaced for 6 h by Hepes-buffered fresh medium, preincubated in air for 1 h.

Production of stably transfected melanoma lines

The protein coding region of VPF_{121} was cloned directionally as a *XbaI*/*KpnI* fragment into the expression vector EBOpLPP (38), by which the sequence was brought under control of SV40 transcriptional elements. This plasmid also codes for the hygromycin phosphotransferase (HPH) gene. The recombinant plasmid, as well as the empty vector, were digested with *ApaI*, thus eliminating some non-essential sequences. The residual 8 kb fragment was transfected into Mel57 cells by calcium phosphate precipitation followed by a 3 min shock with 25% DMSO (31). After 48 h the cells were trypsinized, seeded sparsely in culture flasks and selected in medium with 200 μg/ml hygromycin B (Boehringer Mannheim, Germany). Single resistant colonies were removed by scraping and grown until analysis of VPF expression, and storage of stocks in liquid nitrogen was possible.

Histological analysis of tumours from transfected melanoma lines

Transfected melanoma lines were inoculated in nude mice as described above. Every line was injected in at least two mice on both flanks. Tumour volumes were measured weekly, and growth curves were prepared. Tumours of sizes between 150 and 800 mm^3 were excised 30-60 days after inoculation, and cut into three fragments. One part was formalin-fixed and used to study overall tumour morphology. The other parts were snapfrozen in liquid nitrogen; one was used for RNA isolations, the other to study the vascular patterns and the organization of stroma and extracellular matrix. Cryosections were stained with monoclonal 9F1 which reacts specifically with mouse endothelium (JR Westphal et al, manuscript in preparation), and which is suitable for the examination of tumour vasculature. Also tumour sections were stained with a polyclonal rabbit anti-mouse laminin antiserum (provided by Dr. J. van den Born, Dept. of Nephrology, Nijmegen).

Results

Analysis of VPF mRNA in cultured melanoma cells

The four melanoma cell lines IF6, Mel57, BLM, and MV3 differ in metastatic potential upon s.c. injection in nude mice (23). To determine whether this behaviour correlates with VPF expression, RNA was isolated from these cell lines and examined by Northern analysis. VPF mRNA was readily detectable in RNA from the cell lines BLM and MV3 (Fig. 1) as a major band of 3.7 kb. RNA from the cell lines IF6 and Mel57 showed only very weak hybridization signals with the VPF probe. The highest VPF mRNA levels were thus found in the cell lines BLM and MV3, which were shown in earlier studies to give rise to highly metastatic tumours in nude mice. The other lines, which had a low level of VPF mRNA expression, produced tumours with a much lower metastatic frequency (23).

The observed difference in expression of VPF mRNA is not likely to be caused by a genomic rearrangement or an amplification of the VPF gene. Southern analysis of the genomic DNA isolated from these melanoma lines and from normal human buffy coat showed no obvious differences in intensities of the bands hybridizing with a VPF probe, nor were shifts of bands or extra bands observed in any of the melanoma lines (data not shown).

Alternatively spliced messengers of VPF cannot be distinguished by Northern blotting. To determine whether the melanoma lines differ in the ratio of these mRNA variants, an RT-PCR was performed, which amplified the alternatively spliced region of the

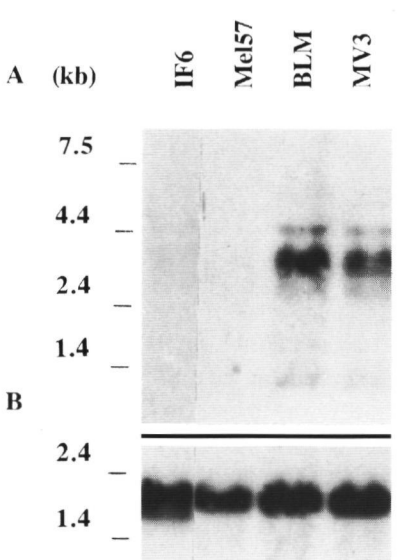

Figure 1. Northern blot analysis of VPF mRNA in melanoma cell lines. 10 μg samples of LiCl/urea-isolated RNA were loaded in each lane. **A:** VPF hybridization; **B:** ubiquitin hybridization. Only relevant parts of the autoradiographs are shown. Positions of RNA size markers are shown on the left.

messenger. RNA from all the melanoma cell lines yielded the same pattern, in that the product from the RNA coding for VPF_{121} was the most prominent (52-70%), followed by the VPF_{165} product (26-42%). The VPF_{189} mRNA was in all cases found to be a minor species (4-8%), while no VPF_{206} mRNA was found in any of the melanoma cell lines (Fig. 2, lanes 2-5). No correlation between the biological behaviour of the melanoma lines and the predominance of the VPF variants produced could therefore be demonstrated. The observed ratio of splice variants does not seem to be specific for melanoma cells, or even for tumour cells, as the same ratio of splice variants was found in RNA from the human lymphoma cell line U937 and in RNA from normal human pericytes (Fig. 2, lanes 6 and 7).

Levels of secreted VPF protein correlate with the VPF mRNA levels

The amount of VPF protein secreted from the melanoma cell lines was determined by culturing the cells in serum-free medium containing heparin, to release also the longer VPF variants from the extracellular matrix (20). The proteins in the conditioned medium were Western blotted and stained with a polyclonal antibody against VPF. In the conditioned media from cell lines BLM and MV3 detectable amounts of VPF were present. Several bands with apparent molecular masses between 17 and 25 kD were identified (Fig. 3), which agree with the expected sizes of non-glycosylated and glycosylated VPF_{121} and VPF_{165} (19,20). VPF_{189} and VPF_{206} (expected around 30 kD) could not be found, supporting the finding that their mRNAs are poorly expressed in

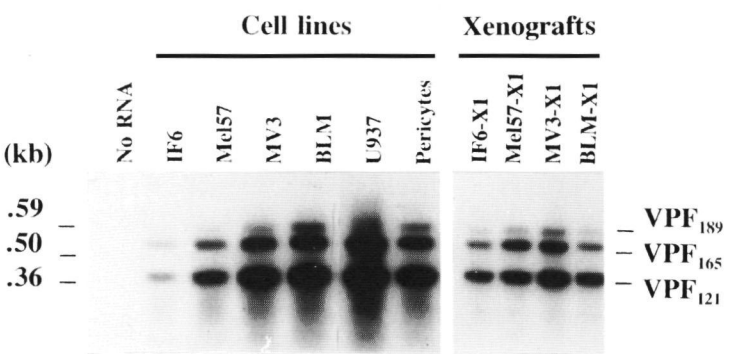

Figure 2. Analysis of VPF mRNA splice variants. RT-PCR was performed on RNAs isolated from various sources, amplifying the alternatively spliced region as indicated in Materials and Methods. Left panel: human cell lines: IF6, Mel57, MV3, BLM: melanoma cells; U937: histiocytic lymphoma cells; Pericytes: from human brain capillaries. Right panel: melanoma xenografts - see also legend to Fig. 4. The left and the right panel are from different gels. Southern blots were hybridized with a VPF probe. Positions of DNA size markers are shown on the left. Expected PCR products: VPF_{121}: 381 bp; VPF_{165}: 513 bp; VPF_{189}: 585 bp; VPF_{206}: 636 bp. Note that no PCR products are expected from mouse VPF mRNA, as the primers do not match the murine sequence completely.

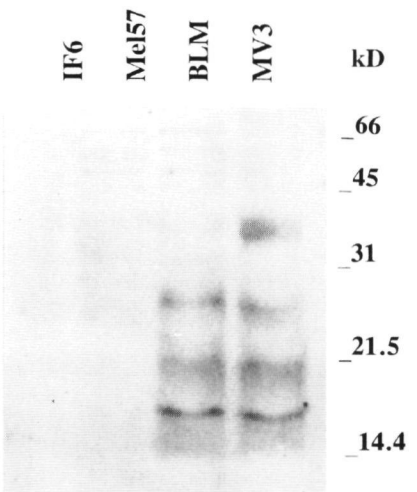

Figure 3. Western blot analysis of VPF in conditioned media from melanoma cells. Confluent cultures of melanoma cells were grown in serum-free medium with 100 μg/ml heparin for 24 h. Precipitated proteins from 0.225-0.7 ml conditioned media (but derived from the same amount of cellular mass) were subjected to SDS-PAGE under reducing conditions, Western blotted, and stained with affinity-purified antiserum against VPF. Molecular mass markers are shown on the right.

melanoma cells (Fig. 2; the band in the MV3 lane of Fig. 3 at 38 kD is probably caused by a dimer of VPF_{121} or by a non-specific reaction rather than by a monomeric form of one of the larger VPF variants). In conditioned media from cell lines IF6 and Mel57 no VPF could be detected, which agrees with the fact that these cells contain low amounts of VPF mRNA (Fig. 1). These results indicate that the levels of VPF mRNA in melanoma cells are reflected in the levels of VPF protein expression.

Analysis of melanoma xenograft RNA

To determine whether VPF mRNA levels change when melanoma cell lines form tumours in nude mice, RNA isolated from melanoma xenografts was examined. The four melanoma lines that were also used in the above experiments (IF6, Mel57, BLM, and MV3) were injected in mice, and the resulting tumours were collected at different time points after injection. All xenografts contained comparable levels of VPF mRNA (Fig. 4), including those derived from the lines IF6 and Mel57, which had hardly detectable levels of VPF mRNA in culture. Scanning of autoradiographs from blots containing both cell line RNAs and xenograft RNAs (not shown) revealed that the average VPF mRNA level in xenografts was 75% of the average level in the cultured cell lines BLM and MV3 (range 30-170%). The small differences between the VPF mRNA levels in xenografts were independent of the parental cell line. The levels of VPF mRNA did tend to decrease with increasing tumour size for those tumours that were derived from the IF6, Mel57, and MV3 lines, but in tumours derived from cell line BLM the opposite effect was seen, namely an increase in the VPF mRNA level with increasing tumour size (Fig. 4). No

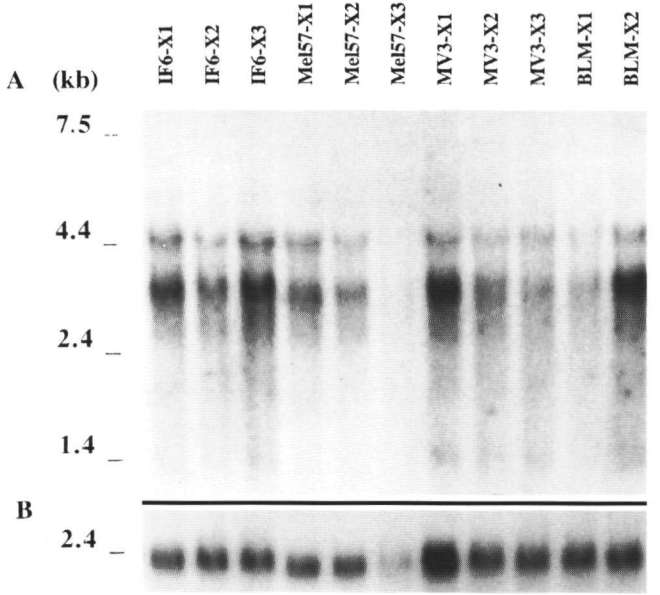

Figure 4. Northern blot analysis of VPF mRNA in melanoma xenografts. 10-16 μg samples of total RNA were loaded in each lane. Parental cell line and number of the xenograft are given with the age and the estimated tumour volume. IF6-X1: 31 days, 180 mm^3; IF6-X2: 31 days, 720 mm^3; IF6-X3: 45 days, 320 mm^3; Mel57-X1: 31 days, 80 mm^3; Mel57-X2: 31 days, 1200 mm^3; Mel57-X3: 45 days, 1340 mm^3; MV3-X1: 31 days, 490 mm^3; MV3-X2: 31 days, 3400 mm^3; MV3-X3: 45 days, 18750 mm^3; BLM-X1: 24 days, 6000 mm^3; BLM-X2: 24 days, 10000 mm^3. **A:** VPF hybridization; **B:** ubiquitin hybridization. Only relevant parts of the autoradiographs are shown. Positions of RNA size markers are indicated on the left.

change in the splicing pattern was found in xenografts: the ratios of alternative splicing products were equal to those in the cultured cell lines (Fig. 2, lanes 8-11).

The VPF messengers found in melanoma xenografts could derive from melanoma cells, but also from host cells within the tumour (stromal cells) or from small amounts of surrounding tissue (e.g. skin). VPF mRNA was indeed found in some murine tissues such as skeletal muscle and heart, but was not detected in normal mouse skin (Fig. 5). These normal mouse tissues are not readily comparable, however, with host tissue within melanoma xenografts. To confirm the melanoma origin of the xenograft VPF mRNA unequivocally, an RT-PCR assay was carried out which discriminates between human and murine VPF sequences. RNA was reverse transcribed and amplified using a set of primers based on sequences common to both the human and murine VPF messenger. PCR products were then digested with *Sty*I, which cleaves the human, but not the murine sequence. As predicted, a 0.35 kb band was found when RNA from murine tissues was

used, whereas two products of 0.25 and 0.1 kb (the latter only slightly visible) arose from human RNA (Fig. 6). Although xenograft RNAs were amplified to different extents, leading to varying amounts of total PCR product, a consistent pattern was seen. In all cases, the human-specific band of 0.25 kb was the most prominent product. The mouse-derived 0.35 kb band was also present in all xenograft RNAs, but its intensity was only 14-25% of that of the human-derived band. The majority of the xenograft VPF mRNA is thus synthesized by the human melanoma cells. For IF6 and Mel57 cells this implies that the expression of VPF is distinctly higher *in vivo* than *in vitro*, and that *in vivo* their VPF expression is no longer different from that in the cell lines BLM and MV3.

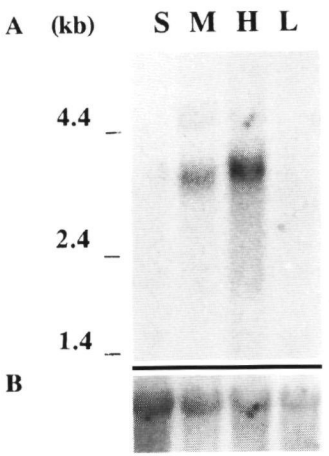

Figure 5. Presence of mRNA for VPF in mouse organs. 10-16 μg samples of total RNA were loaded in each lane. **S:** skin; **M:** skeletal muscle; **H:** heart; **L:** liver. **A:** VPF hybridization; **B:** the 28S signal upon hybridization with a ribosomal probe. Positions of RNA size markers are shown on the left.

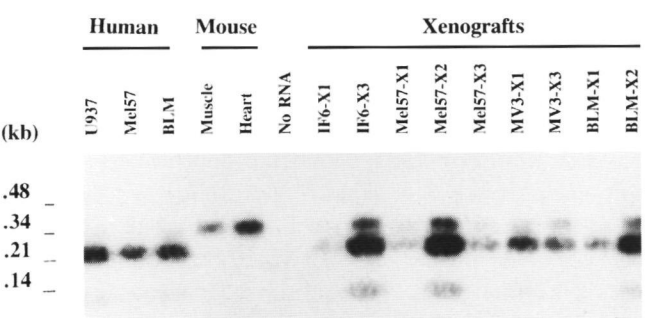

Figure 6. Determination of the species origin of VPF mRNA. RT-PCR followed by *Sty*I-digestion was performed on RNA from various sources to determine the origin of the VPF messengers, as explained in Materials and Methods. Lanes 1-3: Human cell line RNAs; lanes 4-5: Mouse RNAs: skeletal muscle and heart; lane 6: no RNA; lanes 7-15: melanoma xenograft RNAs - see also legend to Fig. 4. A Southern blot was hybridized with a VPF probe, and washed in 2 x SSC at 50°C. Subsequent washing in 0.2 x SSC at 50°C did not change the pattern. Positions of DNA size markers are indicated on the left. Expected fragments from human VPF mRNA: 256 and 90 bp; and from murine VPF mRNA: 343 bp.

Inluencing VPF gene expression

Apparently, VPF mRNA levels are induced in IF6 and Mel57 cells during tumourigenesis in nude mice. To investigate possible mechanisms that could contribute to this induction, Mel57 cells were exposed *in vitro* to factors that may also have been present *in vivo*. After serum deprivation, the cells were incubated for 4 h or 24 h in serum-containing medium, in medium conditioned by mouse fibroblasts (Balb-3T3 cells) with or without serum, or in serum-free media to which a growth factor or cytokine (listed in the Materials and Methods section) was added. No increase in the VPF mRNA levels was found (not shown). IF6 cells also did not respond to addition of serum or Balb-3T3-conditioned medium. Hence, growth factors or cytokines present in serum or fibroblast-conditioned medium, or added separately, apparently do not augment VPF messenger levels under these conditions. When Mel57 cells were cultured in low oxygen tension for 6 h or 24 h, however, a dramatic increase in the VPF messenger content was found (Fig. 7). After reoxygenation of the flasks these levels started to decrease: within 3 hours a reduction of 50% was observed (Fig. 7, lanes 5 and 6). No effect on the VPF mRNA levels was seen when the pH of the medium was varied between 7.2 and 8.1 (Fig. 7, lanes 1 and 2), which excludes the possibility that pH differences due to the hypoxic treatment caused the observed induction. In IF6 cells cultured under low oxygen tension for 24 h, a dramatic induction of the VPF mRNA level was found as well (not shown).

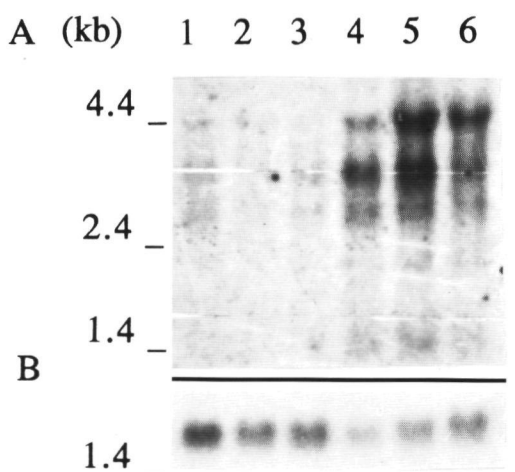

Figure 7. Induction of VPF mRNA in Mel57 cells by hypoxia. Cells were cultured under nitrogen, as described in Materials and Methods. At the end of the incubations, oxygen levels in the gas phase were 0.5-1.0% and Po_2 values in the media were 2.8-3.0 kPa. Control cells were incubated in air/5% CO_2 in media of different pH. Final pH values of the media are indicated. 8-22 μg samples of guanidinium chloride-isolated RNA were loaded in each lane. Lane **1:** air/CO_2, pH 8.1; lane **2:** air/CO_2, pH 7.2; lane **3:** N_2-flushed and 5 h in air/CO_2, pH 7.5; lane **4:** 6h in N_2/CO_2, pH 7.8; lane **5:** 24 h in N_2/CO_2, pH 7.5; lane **6:** 20 h in N_2/CO_2 and 3 h in air/CO_2, pH 7.4. **A:** VPF hybridization; **B:** ß-actin hybridization. Ubiquitin was not used as a control, since the expression of this gene may be altered in stress situations. Positions of RNA size markers are shown on the left.

The absolute induction of VPF messenger levels reached in Mel57 and IF6 cells after 24 h of hypoxia cannot be measured, as hybridizing bands are hardly visible in the uninduced state. The maximally induced VPF mRNA levels in Mel57 and IF6 cells were 1.3-fold and 2-fold the already high basal level in MV3 cells, respectively. The high level of VPF mRNA in MV3 cells could further be increased by a factor of 3 when cultured in low oxygen levels (not shown). These results show that hypoxia increases VPF mRNA levels in melanoma cells such that cell lines with distinctly different levels under normal conditions reach comparable levels under conditions of low oxygen tension. Relative levels of VPF splice variants were analyzed, and found unaltered upon hypoxic shock in Mel57, IF6, and MV3 cells (not shown).

Production of a VPF-overproducing transfectant melanoma line

To determine whether the pattern of VPF expression - constitutive *versus* inducible - actually makes a difference in the process of angiogenesis, and perhaps metastasis, during the development of the melanoma xenografts, we transformed a VPF inducible line into a VPF constitutive line by stably transfecting a construct containing the protein coding region of VPF_{121}, the VPF variant most abundantly expressed in melanoma cells (Fig. 2), into cell line Mel57. The resulting transfectants should have a constitutive VPF expression (as in lines MV3 or BLM) instead of an inducible VPF expression. As a control Mel57 cells were also transfected with vector DNA without a VPF sequence. A number of stable tranfectant lines was examined for expression of recombinant VPF RNA, which is easily recognizable as it is much shorter than endogenous VPF mRNA (see Fig. 8). The transfectant line I-3 had the highest level of recombinant VPF RNA, and was used for further study. Medium conditioned by these cells contained clearly detectable levels of non-glycosylated and glycosylated VPF_{121}, visible as bands of 17 and 20 kD on a Western blot stained with polyclonal anti-VPF. The vector-transfected line E2, which did not show any signs of (recombinant) VPF RNA or protein expression (Fig. 8), was used as control in further experiments.

VPF-transfected line I-3, control transfected line E2 and the parental cell line Mel57 were injected into nude mice. The tumours that developed after inoculation of line I-3 still expressed the 1 kb recombinant VPF RNA, but the endogenous 3.7 kb VPF messenger was detectable in these tumours as well (Fig. 8). The upregulation of VPF mRNA was only a minor contribution to the total amount of VPF RNA produced in these tumours: the band intensities of VPF mRNA were only about 50% of the intensities of the recombinant VPF RNA bands. Tumours from control transfected line E2, as expected, had elevated levels of VPF mRNA but did not show recombinant VPF RNA. Tumours from line E2 did express the vector-encoded hygromycin phosphotransferase RNA (not shown).

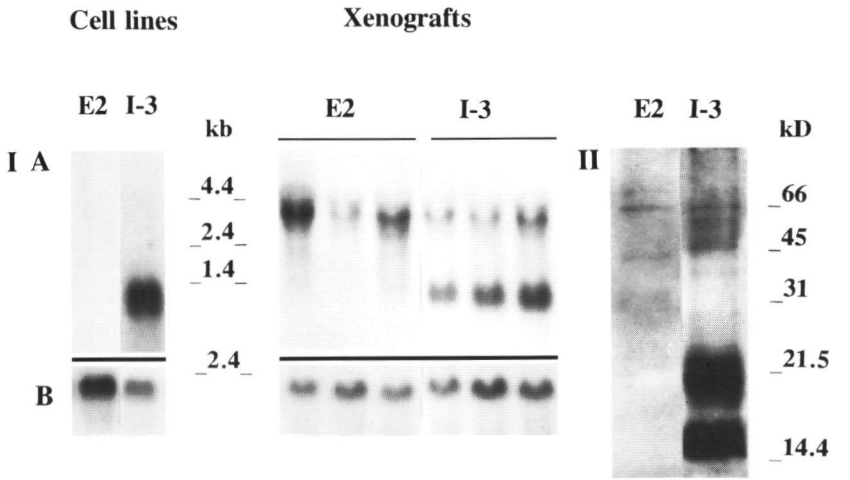

Figure 8. Expression of recombinant VPF RNA and protein by transfected melanoma lines. **I**: RNA isolated from control- (E2) and VPF (I-3)-transfected cell lines (left panel, 10-15 μg of RNA loaded in each lane) and from their respective xenografts in nude mice (middle panel, 22-30 μg of RNA loaded in each lane). **IA:** VPF hybridization (with endogenous VPF messenger bands at 3.7 kb, and recombinant VPF RNA bands at 1 kb); **IB:** ubiquitin hybridization. Only relevant parts of the Northern blots are shown. **II:** Proteins from 0.2 ml of serum-free conditioned media of transfected lines E2 and I-3 were acetone precipitated, electrophoresed on SDS-PAGE, electroblotted and stained with crude antiserum against VPF. Specific staining for VPF_{121} is observed at 17-20 kD. Also some non-specific staining of higher molecular mass bands is visible in both lanes. Positions of molecular mass markers are indicated on the right.

Vascular architecture in transfectant xenografts

Tumours from the parental and the transfected cell lines did not differ significantly in growth rate, nor did they differ in their extent of necrosis (as judged from HE-stained cross sections). Staining of cross sections with the monoclonal antibody 9F1, which reacts specifically with mouse endothelium, showed that the vascular pattern in tumours from VPF-transfected line I-3 was clearly different from the pattern in tumours from the control lines. Tumours from control lines Mel57 and E2 had similar vascular patterns. Typically, in tumours from control lines blood vessels always appeared separately. Large vessels with lumina were visible, as well as smaller microvessels and capillary sprouts in which no lumen could be observed upon light microscopy. Between different regions within one tumour differences in vessel density did occur: some tumour parts were well vascularized and viable, whereas other parts were poorly vascularized and highly necrotic. In regions with a low density of vessels, as in the section shown in Fig. 9A, vessels were surrounded by a layer of viable tumour cells while at a greater distance tumour tissue was necrotic. Tumours from VPF-transfected line I-3 had quite a different

arrangement of blood vessels (Fig. 9B). Vessels formed a dense network around nodules of tumour cells. The endothelial staining in cross sections appeared to be largely continuous, suggesting that there is a three-dimensional plexus of blood vessels surrounding the tumour nodules. Within the tumour nodules very few or no blood vessels were present, often leading to viable layers of tumour cells surrounding necrotic centres.

Staining for the extracellular matrix component laminin showed that in tumours from both the control lines and the VPF-overproducing line tumour nodules were surrounded and separated from each other by stromal septa (Fig. 9, C and D). However, on the average the tumour nodules in the line I-3 tumours were larger than those in control tumours, and the stromal component consisted for a large part of endothelial cells (Fig. 9B), unlike the situation in control tumours, in which only single blood vessels were seen (Fig. 9A). The change in the pattern and level of VPF expression has therefore dramatically changed the vascular architecture in the tumours from this transfectant melanoma line.

Discussion

The initial goal of this investigation was to determine whether the level of VPF expression in several human melanoma lines correlated with the metastatic potential of these lines in nude mice. We did observe this correlation in cultured melanoma cells: lines IF6 and Mel57, which produce rarely or slowly metastasizing tumours, had much lower levels of VPF mRNA and secreted less VPF protein than lines BLM and MV3, which develop into highly metastatic tumours in mice (23, JR Westphal et al; manuscript in preparation). However, these differences in VPF expression were no longer observed *in vivo*: tumours from all four melanoma lines had high levels of VPF mRNA, produced mainly by the melanoma cells. Therefore the highly metastatic melanoma lines have a constitutive VPF expression which apparently is not dependent on the extracellular environment, while the less metastatic lines can have the same level of VPF expression, but only if it is induced by external factors.

The upregulation of VPF expression in IF6 and Mel57 cells *in vivo* might have been caused by protein factors released by e.g. stromal cells. In several cell lines, induction of VPF expression by transforming growth factor ß and platelet-derived growth factor BB has been demonstrated (39-41). We did not find any induction of VPF mRNA by various growth factors or cytokines, serum, or conditioned media of fibroblasts in the melanoma cells IF6 and Mel57. We cannot exclude, however, the possibility that other growth factors, or combinations of such factors can cause an increased VPF expression in these cells.

One of the mechanisms that may lead to enhanced VPF expression *in vivo* is hypoxia, since a dramatic upregulation of VPF messenger levels was evident after incubation of Mel57 and IF6 cells under low oxygen pressure. VPF expression was already known to be induced by hypoxia in glioma and hepatoma cells (14,42), and also the expression of a

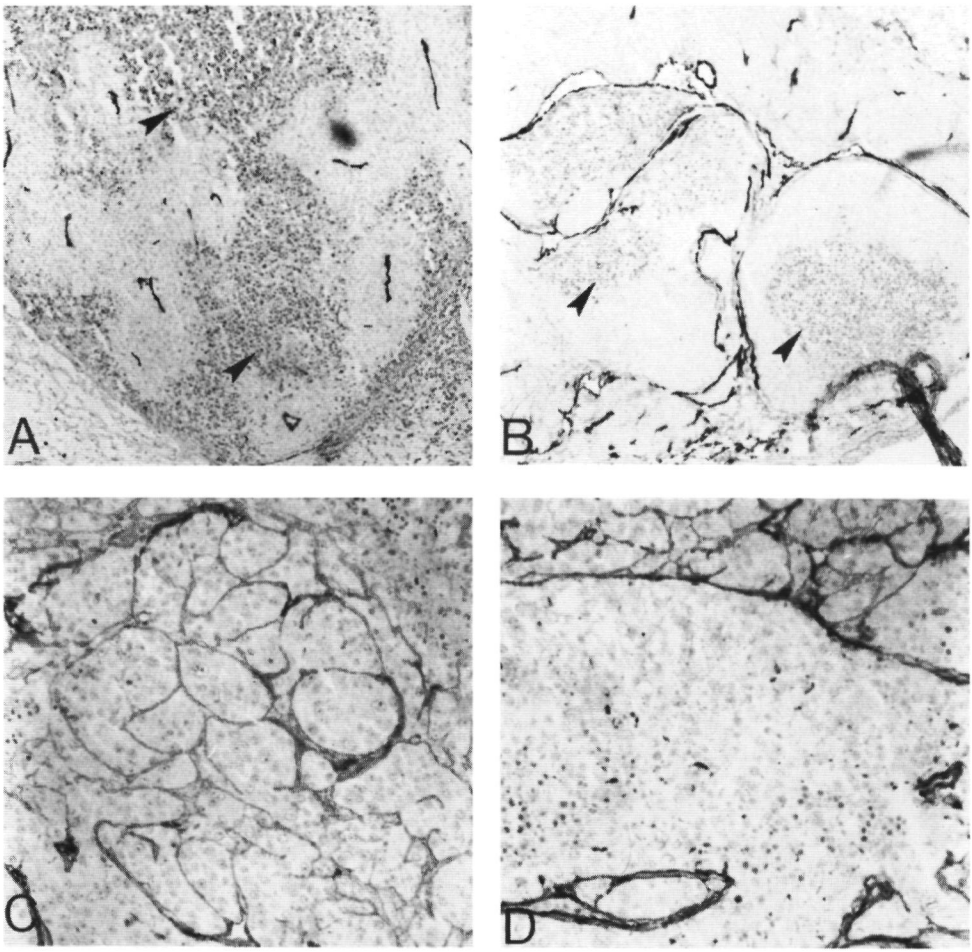

Figure 9. Visualization of tumour vessels and laminin in tumours from transfected melanoma lines. Cross sections were stained with monoclonal 9F1 for endothelium/blood vessels (**A** and **B**), magnification 45x, or with rabbit anti-mouse laminin (**C** and **D**), magnification 225x. **A** and **C:** cross sections of line E2-derived tumours, **B** and **D:** cross sections of tumours from VPF-transfected line I-3. Arrowheads point to areas of necrosis.

number of other genes is induced by hypoxia. The intracellular mechanisms responsible for this kind of induction are only beginning to be understood (43,44). There is evidence that an intracellular haeme protein is involved in hypoxia-induced expression of both VPF and erythropoietin, and this effect of hypoxia on glioma and hepatoma cells is mimicked by cobalt chloride (42). VPF mRNA levels in Mel57 cells were also upregulated by cobalt chloride (not shown), suggesting a regulatory mechanism that is common to many

cell types. In MV3 cells, which have a higher expression of VPF mRNA than Mel57 and IF6 cells when cultured under normoxic conditions, VPF mRNA induction by hypoxia was relatively small. These cells may have undergone an irreversible switch to a high VPF producing phenotype, which would make them less sensitive to VPF-inducing mechanisms.

Hypoxia is thought to be one of the major causes of tumour necrosis. *In situ* hybridization studies have shown that VPF mRNA levels were highest around necrotic sites in glioblastoma lesions, suggesting an involvement of hypoxia in the regulation of VPF expression (14,15). However, in other tumour types most tumour cells produced VPF mRNA in abundance, with only slightly increased levels being found adjacent to necrotic areas (45). In our study we found two sets of melanoma lines, one in which VPF mRNA levels were upregulated by hypoxia, and a second in which VPF mRNA levels were only slightly affected by oxygen tension. Further evidence for the assumption that hypoxia is the major trigger for the VPF mRNA upregulation observed by us *in vivo* can only be provided by *in situ* hybridization or by immunohistochemistry. These experiments are in progress in our laboratory.

A relation between tumour angiogenesis and metastasis has been postulated on theoretical grounds, and has also been demonstrated in practice in several tumour types including melanoma (8,46-49). Since VPF is a potent angiogenic factor, it might also play an important role in the development of the metastatic phenotype of melanoma xenografts. Although the xenografts of all melanoma lines examined showed comparable VPF expression, this expression in the less metastatic lines IF6 and Mel57, had to be upregulated first *in vivo*. A lower expression level of VPF in early IF6 and Mel57 xenografts might result in a delayed development of the vascular bed compared with BLM and MV3 xenografts, which might eventually hamper the opportunities for dissemination. To separate the effect of VPF expression on the vasculature from the effects of other factors (differentially) produced by the various melanoma lines, it is necessary to manipulate VPF expression in one of the melanoma lines.

By transfection into cell line Mel57 of a VPF_{121} sequence, we were able to produce a Mel57-derived cell line that had a constitutively high expression of VPF. In this way it was possible to study the biological behaviour of tumours from melanoma lines with an inducible or a constitutive VPF expression, in an otherwise unchanged genetic background. The arrangement of the tumour vasculature indeed turned out to be quite different in tumours resulting from the constitutive VPF-expressing transfectant line. In an other study (50), two types of vascular branching in tumours were distinguished. "Tumour-penetrating branches of variable diameter" were found in some tumours, and "lateral surface branches that formed an arborizing and anastomosing plexus of interconnecting vessels" were found in other tumours. The vasculature in tumours from the control melanoma lines resembled the first type, whereas in tumours from the VPF-transfected line the vasculature appeared to be more like the second type. An altered VPF expression obviously is sufficient for a switch from one vascular type to the other.

Studies from others, in which Chinese hamster ovary cells or HeLa cells were transfected with VPF expression constructs, showed that overexpression of VPF can lead to a growth advantage in nude mice, combined with higher angiogenic activity (51,52). The tumours from the VPF-transfected melanoma line used in our study did not show such behaviour: they did not have a faster growth rate, and angiogenesis was qualitatively rather than quantitatively different. Obviously, angiogenesis is too complex a process to have its outcome predicted by the level of VPF expression only. Similarly, the vasculature in xenografts from transfectant line I-3 might have been expected to be very much like that in xenografts from other constitutively VPF-producing melanoma lines such as BLM and MV3, but this was not at all the case (JR Westphal et al; manuscript in preparation). VPF expression in line I-3 was probably much higher than in lines BLM and MV3, and transfectant lines with a lower recombinant VPF expression than line I-3 should therefore be studied. Even then, VPF is probably not the only factor relevant to angiogenesis in these tumours; a number of other factors directly or indirectly involved in angiogenesis may be differentially expressed between BLM and MV3 on one hand and Mel57 and its transfectants on the other hand. Differences in expression of various integrins and of proteins involved in the plasminogen activator pathway have indeed been shown between some of these melanoma lines (24,25). The vascular phenotype in any tumour will be the result of a large number of factors influencing angiogenesis, but our study shows that VPF is at least one of the important factors governing angiogenesis in these melanoma xenografts.

Further study has to be undertaken to completely understand the way the aberrant vascular phenotype in tumours from line I-3 evolves. Furthermore, it is an interesting question whether the altered VPF expression pattern leads to a different degree of vascular permeability for e.g. fibrinogen or for labelled tracers (50). Experiments addressing this issue are currently in progress.

We also hope to answer the initial question whether VPF expression is important for the metastatic potential of melanoma xenografts in nude mice. However, as the constitutive expression of VPF by the Mel57-derived cell line I-3 led to a vascular phenotype unlike that of other constitutive VPF-producing lines such as BLM and MV3, the metastatic frequency of line I-3-derived tumours may not simply be expected to increase to the frequencies found for tumours from lines BLM and MV3. Although it is still of interest to study the metastatic potential of tumours from line I-3, xenografts from other transfectant melanoma lines with expression levels of recombinant VPF lower than in line I-3 perhaps will provide a better basis for comparing the metastatic potential with lines BLM and MV3. Therefore other transfectant lines will need to be included in future studies on the correlation between VPF levels, vascular pattern, and metastasis.

Acknowledgements

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Chapter 4

Functional Analysis of the Tumour Vasculature in Xenografts of VPF-Transfected Human Melanoma Cell Lines

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This Chapter is to be submitted for publication.

Vascular permeability factor (VPF) is an important mediator of vascular development in tumours. Some human melanoma cell lines have a low VPF expression level in culture but this level is upregulated when growing as a tumour in nude mice. Other melanoma lines have a constitutively high VPF expression. To compare the biological behaviour of tumours with these two expression patterns, a human melanoma cell line with an inducible VPF expression was transfected with VPF expression constructs. In this way several lines were obtained that constitutively produce either the soluble VPF_{121} or the matrix-associated VPF_{189} variant at levels of 4 to 30 times the VPF level in mature tumours derived from the parental line. The recombinant VPF RNA, which lacks most of the 5' non-coding sequences present in the endogenous VPF mRNA, was much more efficiently translated than the endogenous messenger. Upon injection in nude mice all VPF-transfected lines developed tumours with aberrations in vascularization and in distribution of matrix components. In tumours from the parental line or from vector-transfected lines blood vessels were found as separate units randomly penetrating the tumour cell mass. Small groups of tumour cells were found to be surrounded by a thin layer of matrix material containing laminin and heparan sulphate proteoglycan. In VPF-overproducing tumours, on the other hand, blood vessels were almost exclusively found in large stromal septa separating large nests of tumour cells. These septa stained intensely with a marker for endothelial cells, but also contained other cell types and extracellular matrix components that were not found within the tumour nests. Furthermore, in the tumours from VPF-transfected lines, blood vessels were hyperpermeable for an i.v. injected protein tracer. However, this enhanced permeability did not yield a more extensive extravascular fibrin matrix. We could not demonstrate a significant difference between the metastatic potential of tumours from the VPF-transfected lines and from the control lines. We conclude that the pattern or the level of VPF expression has a profound impact on the development of the vascular pattern, on vascular permeability, and on matrix distribution in these melanoma xenografts.

Introduction

Angiogenesis, the formation of new blood vessels from existing vessels, is a process essential for tumour growth. Tumours without a vascular bed do not grow beyond a diameter of 1-2 mm^3 (1). In addition, tumour metastasis is dependent on angiogenesis, as blood vessels provide the most important escape route for disseminating tumour cells (1-3). In recent studies a correlation between tumour vascularization and metastatic risk has indeed been demonstrated (4-8). Outgrowth and malignant behaviour of a tumour, therefore, is accompanied by rapid development of the tumour vasculature, and, perhaps because of this, tumour blood vessels often have a chaotic architecture and some other characteristics distinct from normal blood vessels (reviewed in 9,10). The permeability of the tumour vasculature for macromolecules is often higher than in normal blood vessels (9,10), although this permeability differs significantly between various tumour types (11). Vascular permeability has also been shown to be lower in tumour-penetrating vessels than in a vascular plexus at the tumour-host interface (12,13). Tumour blood vessels are often prone to intravascular coagulation, leading to deposition of fibrin and the occurrence of intravascular thrombi (14).

The development of the tumour vascular bed is thought to be mediated by a number of tumour-derived angiogenic growth factors and cytokines (reviewed by 2,15, and 16,17). Among these factors, vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is gaining attention, as it has become clear that it is an essential factor in tumour angiogenesis and tumour growth (18-20). The main targets of VPF are endothelial cells, which are almost the only cells expressing VPF receptors (21-25). The capacity of VPF to induce angiogenesis is probably due to the induction of a variety of functions in the endothelial cell, including proteolytic activity, chemotaxis, and proliferation (21,26-28). VPF may also be responsible for other characteristics of the tumour vasculature, like vascular hyperpermeability (29,30) and intravascular coagulation (via the induction of tissue factor expression on endothelial cells, 31). By enhancing tumour vessel permeability, VPF may contribute indirectly to angiogenesis, as fibrinogen leaking from the vessels can form an extravascular fibrin matrix serving as a substrate for endothelial outgrowth (32).

In a previous report we described that a human melanoma cell line, Mel57, expressed low VPF messenger and protein levels in culture, but contained elevated levels of VPF mRNA after injection into nude mice and the formation of a tumour (33). Hypoxia was shown to be a likely mediator of this VPF upregulation. The tumours from this melanoma line had a characteristic vascular pattern, with blood vessels penetrating the tumour parenchyma in an apparently random fashion. This melanoma line was engineered into a line constitutively expressing VPF by transfection. Xenografted to nude mice this line developed into tumours with a vascular pattern distinct from parental line tumours: these tumours consisted of several tumour cell nodules devoid of blood vessels, separated by robust stromal septa very rich in blood vessels (33).

In the present study we have extended these findings by generating a panel of VPF-transfected melanoma lines expressing different molecular variants of VPF at different levels. The recombinant VPF RNA, lacking most of the 5' non-coding sequences of the VPF gene, was translated into protein much more efficiently than the endogenous VPF messenger. We further demonstrate that transfectant lines with differing levels of recombinant VPF expression all develop tumours with the same distinct vascular phenotype. Furthermore, the same aberrant vascular pattern was found in tumours from lines overproducing either VPF_{121}, a readily secreted VPF splice variant, or VPF_{189}, a variant that is largely retained in the extracellular matrix of the producing cells (34). In addition, we show that an altered VPF expression pattern changes the distribution of matrix proteins within the melanoma xenografts, and enhances the permeability of the tumour blood vessels for macromolecules. However, the altered VPF expression could not be shown to affect the metastatic potential of the tumours significantly.

Materials and Methods

Cell culture

The human melanoma cell line Mel57 was cultured as previously described (35). Transfected Mel57 cells were cultured in medium supplemented with 200 μg/ml hygromycin B (Boehringer Mannheim, Germany).

Production of stable transfectants

The protein coding regions of VPF_{121} and VPF_{189} were obtained by RT-PCR on RNA from cell line U937, and were subsequently cloned into vector pUC19, as described elsewhere (33). After sequence analysis, the inserts were recloned between the *Xba*I and *Kpn*I sites of the plasmid EBOpLPP (36). Plasmids with VPF_{121} and VPF_{189} inserts, as well as the empty vector, were linearized with *Apa*I, the 7-8 kb fragments were isolated from low melting point agarose gel slices, and were transfected into Mel57 cells by calcium phosphate precipitation (37). Transfected clones were selected in medium with 200 μg/ml hygromycin B, removed by scraping and expanded until analysis of the clones and storage of stocks in liquid nitrogen was possible (see also ref. 33).

Analysis of VPF expression on the RNA and protein level

RNA from cell lines was isolated using guanidinium chloride according to (37), and RNA from tumour xenografts was isolated by disruption in guanidinium isothiocyanate and CsCl centrifugation, as described in (38). Northern analysis and hybridizations with VPF and ubiquitin probes were performed as described earlier (33). To monitor VPF protein secretion cells were cultured for 24 h in serum-free medium lacking hygromycin,

containing 100 μg/ml heparin where indicated. Conditioned media were cleared by centrifugation and used for VPF protein analysis. To correct for differences in cellular density, cells were scraped and lysed, and the cellular protein content was determined with a standard protein assay (Biorad, Veenendaal, The Netherlands). Based on this protein determination, samples of conditioned media derived from equal amounts of cell material were taken and tested in a procoagulant assay (31,39). Similarly, samples were acetone precipitated, run on SDS-PAGE, and analysed by Western blotting and staining with a polyclonal antiserum raised against *E. coli*-produced VPF, as described elsewhere (40).

Xenografts from line Mel57 and transfectants in nude mice

About 2.5×10^6 cells were injected s.c. into BALB/c *nu/nu* mice as described in (35). Every week tumour volumes were estimated. Tumours of between 300 and 1200 mm^3 were harvested and cut into three fragments. One part was formalin-fixed, HE-stained, and used to study overall tumour morphology and to assess the percentage of necrosis. The other parts were snap-frozen in liquid nitrogen: one was used for RNA isolations, the other to study the vascular patterns, the organization of extracellular matrix components, and the spatial distribution of VPF within the tumours (see below). To study vascular morphology in early stages of tumour development, for some lines also smaller tumours were taken (within one week after the occurrence of a palpable tumour), snap-frozen, and stained immunohistochemically. Acetone-fixed 4 μm cryosections were stained with a rat monoclonal, MEC 7.46, directed against mouse vascular endothelium (a gift from Dr. A. Vecchi, Milano, Italy, 41), a rabbit polyclonal against mouse laminin (a gift from Dr. J. van den Born, Dept. of Nephrology, Univ. Hosp. Nijmegen), a goat polyclonal against (human) heparan-sulphate proteoglycan (HSPG, a gift from Dr. L. van den Heuvel, Dept. of Pediatrics, Univ. Hosp. Nijmegen), or with a rabbit polyclonal antibody against human VPF/VEGF (Santa Cruz Biotechnology, Sanvertech, Breda, The Netherlands). For visualization of the immune reactions, alkaline phosphatase-conjugated secondary antibodies were used, except for the anti-HSPG reaction, which was visualized using a peroxidase-conjugated donkey anti-goat antibody. All sections were counterstained with haematoxyllin.

For the analysis of tumour vessel permeability, mice bearing tumours with sizes between 100 and 700 mm^3 were i.v. injected with 0.1 ml of a 3% solution of fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA, 11 moles FITC per mole BSA, Sigma, Brunschwig, Amsterdam, The Netherlands). Tumours were excised 2, 10, or 45 min after the injection of tracer, and snap frozen in liquid nitrogen. Cryosections of these tumours, 4 μm thick, were immediately fixed in 2% formaldehyde solution for 10 min. The distribution of the tracer was shown by fluorescence microscopy. The deposition of extravasated fibrin was analysed in parallel acetone-fixed tumour sections with a goat polyclonal against mouse fibrinogen/fibrin (Nordic Immunological Laboratories, Tilburg, The Netherlands), using a peroxidase-conjugated donkey anti-goat secondary antibody.

Other parallel sections of these tumours were stained for endothelium or HSPG (see above).

Metastasis of Mel57 and transfectant xenografts

To study the time-course and the frequency of metastasis, a number of lines was injected in 10-20 mice. Mice were sacrificed when the tumours reached a volume of between 300 and 2000 mm^3. The lungs were formalin-fixed, and cross-sections were HE-stained and analysed for the occurrence of metastases as described in (35). Scoring was performed by 3 independent observers. The number of metastases found in two cross-sections of both lungs was plotted against the tumour volume. The metastatic frequency was defined as the number of mice having one or more lung metastases divided by the number of mice having a primary tumour.

Results

Selection of melanoma cell transfectants

Mel57 is a melanoma line with a low level of VPF expression in culture, but in which this level is elevated when growing as a tumour in nude mice. A transfectant of this line, producing VPF_{121} constitutively, had quite a different vascular pattern in mouse xenografts compared with the parental line tumours (33). To determine if the level of recombinant VPF expression, or the molecular variant expressed, is of influence on the arrangement

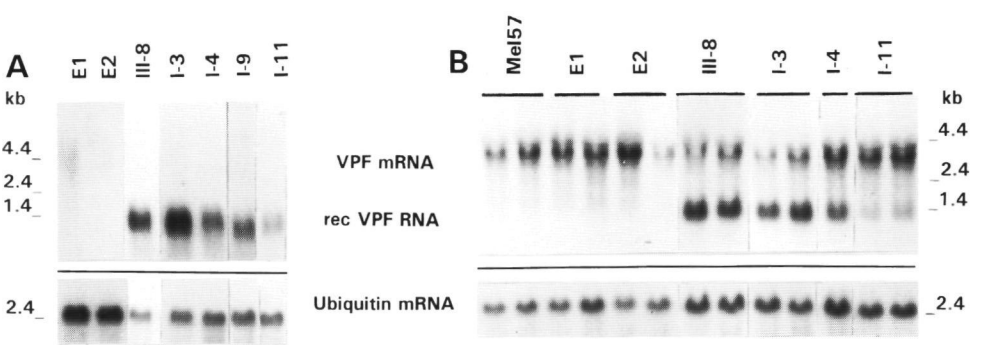


Figure 1. Expression of endogenous and recombinant VPF RNA by melanoma cell transfectants. Northern blots containing RNA from cell lines (**A**) or xenografts in nude mice (**B**) were hybridized with a VPF probe (upper panels) and with a ubiquitin probe (lower panels). Endogenous VPF mRNA is visible as multiple bands around 3.7 kb in tumour RNA only, recombinant VPF RNA is apparent in VPF-transfected lines and their tumours as a band of about 1.0 kb. Mel57: parental melanoma line; E1, E2: lines transfected with vector DNA only; III-8: line transfected with VPF_{189} cDNA; I-3, I-4, I-9, I-11: lines transfected with VPF_{121} cDNA. Positions of length standards are indicated on both sides.

and the functionality of the tumour vasculature, we selected several other VPF_{121}- and VPF_{189}-transfected melanoma lines.

The expression levels of the transfectants were determined by Northern blotting. In RNA from the VPF-transfected cell lines a band of approximately 1.0 kb was seen upon hybridization with a VPF probe, representing the recombinant VPF RNA (see Fig. 1, panel A). The highest levels of recombinant VPF RNA were produced by lines I-3 (expressing VPF_{121}, described before in ref. 33) and III-8 (expressing VPF_{189}). Lines I-4, I-9, and I-11 contained lower but still detectable levels of VPF_{121}-encoding recombinant VPF RNA (see also Table 1). The major endogenous VPF transcript is 3.7 kb in length, but this was undetectable in RNA from cell line Mel57 or its derivatives. Southern blots of genomic DNA from transfected lines, digested with *Hin*dIII, showed multiple bands upon hybridization with a VPF probe, in addition to the bands deriving from the endogenous VPF gene (not shown), indicating that multiple copies of the transfected construct had been integrated.

To confirm that the recombinant VPF RNA levels found in the lines described above correlate with the amounts of active VPF protein secreted, conditioned serum-free media of these lines were harvested. The supernatants were Western blotted and stained with a polyclonal antiserum against VPF. In conditioned media of the parental line Mel57 and the vector-transfected lines E1 and E2 no VPF protein could be detected. Lines I-3, I-4, I-9, and I-11, expressing the RNA encoding recombinant VPF_{121}, secreted detectable amounts of 16 kD and 20 kD proteins (Fig. 2), being the expected molecular masses of the non-glycosylated and glycosylated forms of VPF_{121} (42). Line III-8, expressing the

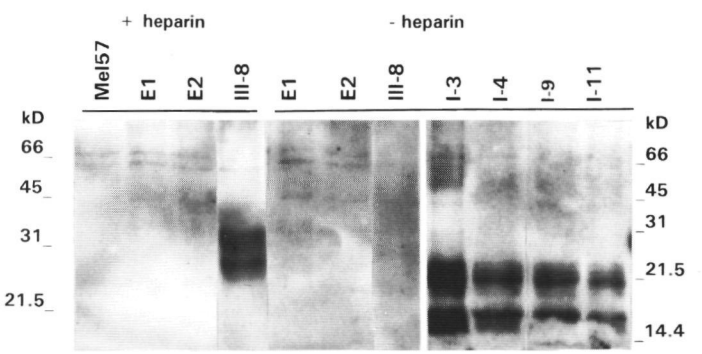


Figure 2. VPF protein expression by Mel57 transfectants. Proteins were precipitated from 0.2-0.4 ml (standardized amounts, see "Materials and Methods") serum-free conditioned media of melanoma cell transfectants, electrophoresed under reducing conditions, electroblotted and stained with a polyclonal antiserum against human VPF. Mel57: parental cell line; E1, E2: lines transfected with vector DNA only; III-8: line transfected with VPF_{189} cDNA; I-3, I-4, I-9, I-11: lines transfected with VPF_{121} DNA. Conditioned media in the four left-most lanes contained 100 μg/ml heparin, conditioned media in the other lanes did not contain heparin. The four right-most lanes were run on a separate gel. Positions of molecular mass standards are indicated for both gels.

RNA for recombinant VPF_{189}, was expected to secrete VPF proteins of a molecular mass of 28 and 31 kD which would need heparin to be released from extracellular matrix (34,42). Indeed, detectable amounts of VPF were only found in the conditioned medium of this line if heparin was added (Fig. 2). The biological activity of the recombinant VPF was tested in a procoagulant assay, measuring the capacity to induce tissue factor expression on endothelial cells. No significant activity was present in the conditioned media of Mel57 and control transfectants, but the lines that produced recombinant VPF were all positive in this assay (not shown). The highest tissue factor level was induced by conditioned medium from line I-3. Although no VPF_{189} was detectable in the heparin-free conditioned medium of line III-8 by Western blotting (Fig. 2), sufficient VPF activity was present in this supernatant to cause a significant increase in the tissue factor level.

The efficiency of translation of recombinant VPF RNA

The endogenous VPF mRNA has a 1 kb 5' non-coding sequence in which a very GC-rich region and several alternative translational start sites are present (43). These features may inhibit the efficiency of translation of the VPF mRNA. The recombinant VPF RNA lacks these sequences: only a stretch of 21 bases of the 5' non-coding sequence of the VPF gene is contained in the recombinant RNA. The efficiency of translation of the recombinant VPF RNA in Mel57 transfectants was compared with that of the endogenous VPF messenger in melanoma line BLM which has a constitutively high VPF expression (33).

In transfectant cell line I-9 no endogenous VPF mRNA was detectable; its level of recombinant VPF_{121}-encoding RNA (1 kb) was about the same (in a molar ratio) as the level of endogenous VPF mRNA in melanoma line BLM (3.7 kb, Fig. 3, panel A). The VPF mRNA in line BLM was previously shown to encode predominantly VPF_{121}, along with some VPF_{165} (33). The VPF protein secreted by cell line BLM was barely detectable on Western blots (bands representing VPF_{121} and VPF_{165} can be seen between 16 and 24 kD), whereas the VPF_{121} bands produced by line I-9 are clearly visible (Fig. 3, panel B). Scanning of bands on autoradiographs from several Northern and Western blots revealed that VPF is 16 to 40-fold more efficiently produced from recombinant VPF RNA in line I-9 than from the endogenous VPF messenger in line BLM. In estimating the expression levels of recombinant VPF protein in tumours we have taken this higher efficiency of translation into account (see below, and Table 1).

Expression of recombinant VPF in xenografts

Transfected melanoma lines positive for recombinant VPF expression, as well as the parental line Mel57 and vector-transfected lines E1 and E2, were injected into nude mice, and from the resulting tumours RNA was isolated. Northern blotting of these RNAs showed that recombinant VPF RNA was present in tumours from the VPF_{121}-producing

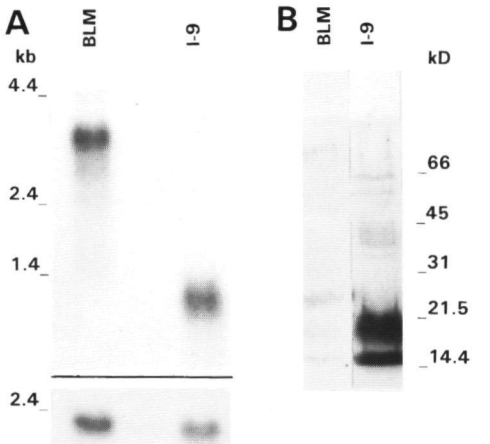


Figure 3. Analysis of the relative translation efficiency of recombinant VPF RNA. **A**: Northern blot with RNA from melanoma cell line BLM and from VPF_{121}-transfected cell line I-9, hybridized with a VPF probe (upper panel) and a ubiquitin probe (lower panel). Length markers are indicated on the left. **B**: Serum-free conditioned media containing 100 μg/ml heparin from melanoma cell line BLM and transfectant cell line I-9, were electrophoresed on SDS-PAGE under reducing conditions, electroblotted, and stained with polyclonal antiserum against VPF (blocked before use with 3 volumes of heat-denatured FCS to reduce non-specific background). Loaded were 285 μl of line I-9-conditioned medium (as in Fig. 2) and 500 μl of BLM-conditioned medium (derived from 3 times more cells than the sample from line I-9). Molecular mass standards are indicated on the right. VPF_{121} bands are visible at 16 and 20 kD; cell line BLM produces VPF variants of 121 and 165 amino acids, which are faintly visible as bands between 24 and 16 kD.

lines I-3, I-4, and I-11, and from the VPF_{189}-producing line III-8 (Fig. 1, panel B). In these tumours, as well as in the tumours from vector-transfected lines E1 and E2, the vector-encoded RNA for hygromycin phosphotransferase was also still expressed (not shown). Line I-9 produced only one small tumour after the first series of four injections, and was not included in further experiments.

In all xenografts the levels of endogenous VPF mRNA were increased, as expected (33). However, in tumours from the lines expressing the highest levels of recombinant VPF RNA (I-3 and III-8) the levels of endogenous VPF mRNA were about half of the level in line Mel57 tumours. This suggests that VPF might - possibly through inducing angiogenesis and preventing hypoxia - inhibit the expression of its own gene *in vivo*. The relative levels of recombinant VPF RNA and endogenous VPF mRNA in the various transfected cell lines *in vivo* are summarized in Table 1.

Extrapolation of the higher efficiency of VPF production from the recombinant VPF RNA than from the endogenous messenger (see above) to the *in vivo* situation, suggests that the levels of recombinant VPF protein expressed by the VPF-transfected tumours greatly exceed the VPF levels expressed by the parental line tumours. The level of recombinant VPF RNA in line I-3 *in vivo* is only 1.8-fold its endogenous messenger level. The amount of recombinant VPF protein produced would be, however, 13 to 33-fold the amount of VPF produced by Mel57 tumours. Tumours from the lower expressing lines I-4 and I-11, are still predicted to express 4 to 10-fold the VPF levels in Mel57 tumours (Table 1).

Line	rec VPF RNA *in vivo*		end VPF mRNA *in vivo*		predicted VPF expression level
Mel57	-		1	(0.5-1.2)	1
E1	-		0.7	(0.6-0.8)	0.7
E2	-		2.1	(0.7-3.8)	2.1
I-11	0.19	(0.15-0.25)	1.1	(0.9-1.2)	4 - 9
I-4	0.27	(0.17-0.38)	0.58	(0.5-0.7)	5 - 11
I-3	0.82	(0.53-0.97)	0.47	(0.2-0.6)	14 - 33
III-8	0.78	(0.74-0.80)	0.44	(0.3-0.5)	13 - 32

Table 1. Relative levels of recombinant (rec) VPF RNA and endogenous (end) VPF mRNA in (transfected) melanoma lines *in vivo*, and predicted expression levels of VPF protein *in vivo*. VPF band intensities after Northern hybridizations were quantified by densitometry. Values were corrected for the amount loaded by dividing the VPF intensities by the ubiquitin intensities. The average value for VPF mRNA calculated for Mel57 tumours was defined as 1. The ranges of values obtained with different tumours from one line are given in parenthesis. Estimates of VPF protein expression levels in the tumours were made by considering the contribution from endogenous VPF mRNA and from recombinant VPF RNA in the tumours, assuming a 16-40-fold higher translation efficiency of the latter RNA. The predicted VPF protein expression level in Mel57 tumours was defined as 1.

Growth of transfected melanoma xenografts

The growth curves of at least 4 tumours from each of the transfectant melanoma lines and from the parental line Mel57 were compared, and were found not to differ significantly. Only tumours from line III-8 had a somewhat higher growth rate *in vivo* than the other lines, but the cells from this line grew faster *in vitro* as well (not shown). Therefore it is unlikely that the increased rate of tumour growth was caused by the enhanced expression of VPF. The percentages of tumour necrosis were determined from formalin-fixed and HE-stained sections. Again, no significant differences between the lines were discovered (data not shown). All lines produced some tumours with extensive necrosis (up to 60-80% of cross-sections), while most lines (I-11, I-4, III-8, E1, and E2) also developed tumours with low necrosis percentages (10-40%). The higher levels of VPF expression in the VPF-transfected lines thus did not cause faster tumour growth or a higher ratio of viable cells, which might have been expected if the increased level of VPF had increased the density of tumour blood vessels.

All VPF-transfected melanomas have an altered vascular pattern

Staining of Mel57 xenograft sections with the vascular marker MEC 7.46 showed blood vessels of different sizes scattered in an apparently unorganized fashion over large parts of the tumours. This vascular phenotype also developed in vector-transfected xenografts E1 and E2 (Figs. 4a, 4b, 5a). Many necrotic sites with little or no vasculature were present in these tumours as well (not shown here, but see also (33), in which an identical vascular pattern was described).

Staining of xenograft sections from VPF-transfected lines with MEC 7.46 showed a vascular pattern very different from that in the control tumours. These tumours were divided into multiple nodules separated by stromal septa. Mostly, the tumour cell nests were devoid of endothelial staining, and developed a central necrosis. The stromal septa, however, showed intense endothelial staining (Figs. 4c-e, 5b). Endothelial cells extended through large parts of the septa, tending to surround most of the tumour cell nodules. However, the stromal septa also contained cells not staining with the endothelial marker. A double layer of endothelium was often observed within the septa (Figs. 4e and 5b), either suggesting hyperproliferation of endothelial cells, or compression of larger vessels by the (expanding) tumour mass. Cavities lined by endothelial structures were detectable, which probably acted as functional blood vessel lumina (Figs. 4c-e, 5b). This was confirmed by tracer studies (see below), since immediately after i.v. injection tracer was predominantly found in and around such cavities within the stromal septa.

Line I-3 expressed the highest levels of VPF_{121}, whereas lines I-4 and I-11 expressed lower, but still constitutive, levels of VPF_{121} (see Table 1). Yet, the lower levels of VPF expression in the latter lines still caused the development of the same vascular phenotype as found in line I-3 tumours.

In xenografts derived from line III-8, which overexpresses the extracellular matrix-bound variant VPF_{189}, the vascular patterns (Fig. 4e) were similar to those found in the lines overproducing VPF_{121} (I-3, I-4, and I-11). Stromal septa were often wider than observed in line I-3, I-4, or I-11 tumours: especially at the junctions between several septa, enclosed by two layers of endothelial cells, a layer of non-endothelial stromal cells was often found, presumably consisting of fibroblasts (Fig. 4e, right hand side). Endothelial staining in line III-8 xenografts was exclusively found in the stromal septa. The morphological similarity demonstrates that also the heparin-binding VPF_{189} variant has an effect on vascular development *in vivo*, and that there is no significant difference between the biological effect of the diffusible and the matrix-bound VPF variants.

The differences in the vascular phenotype between tumours from control lines and from VPF-transfected lines were already established in very small tumours (< 20 mm^3, isolated within one week after the occurrence of a palpable tumour). A line E1 tumour with dimensions of 1.5 x 0.8 mm already showed a few tumour-penetrating vessels, whereas a line I-3 tumour with dimensions of 3 x 2 mm already demonstrated a nodular phenotype with strong endothelial staining in the stromal septa (not shown).

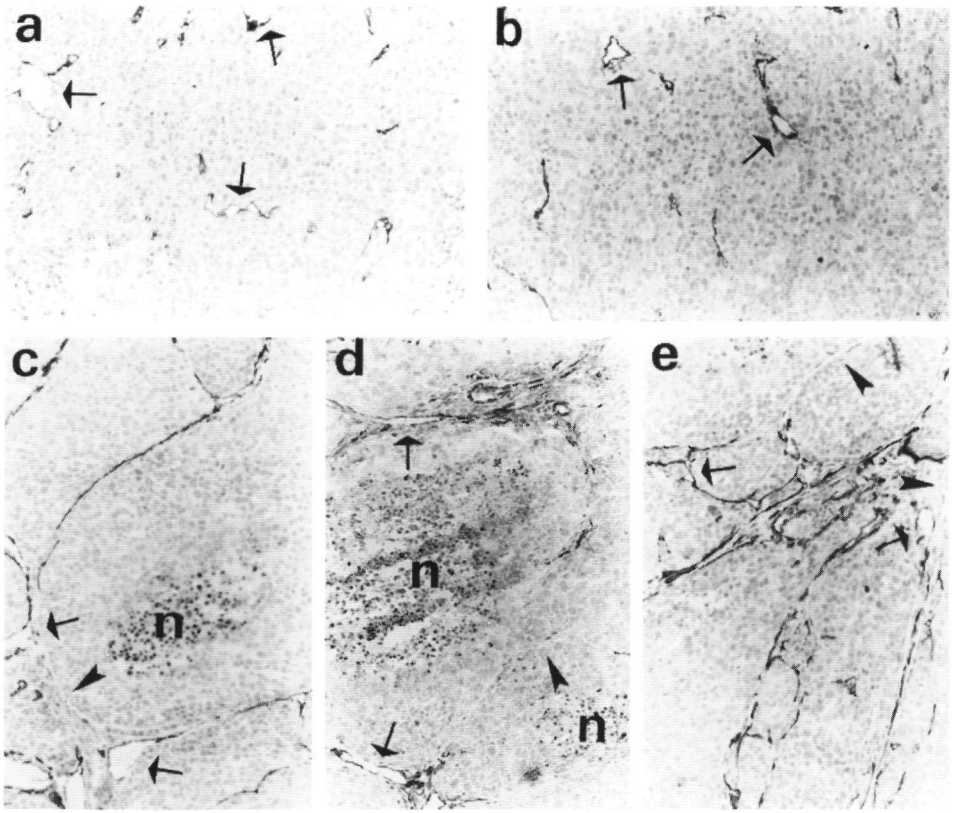


Figure 4. Endothelial staining of sections of tumour tissue originating from several melanoma (transfectant) lines. The monoclonal MEC 7.46 was used to locate the murine endothelium in tumours from the following lines: **a:** Mel57, parental melanoma line; **b:** E2, vector-transfected line; **c:** I-4; **d:** I-11; **e:** III-8. Areas of necrosis are marked "**n**". Blood vessels are marked by **arrows**, and apparent stromal structures that do not stain with the anti-endothelial antibody (non-endothelial stroma) are marked by **arrowheads**. Magnifications: 80x.

Distribution of extracellular matrix

Vascular endothelium is generally lined by a basal lamina consisting of extracellular matrix. In addition, melanoma cells may produce and deposit an extracellular matrix as well. To study the distribution of extracellular matrix components in the xenografts we used antibodies directed against laminin and heparan-sulphate proteoglycan (HSPG). Both antibodies demonstrated a finely branched network of extracellular matrix in the tumours from vector-transfected line E1 (see Figs. 5c and 5e), which was also apparent in tumours from lines Mel57 and E2 (not shown). A similar pattern was seen upon staining with an

anti-collagen IV antibody (not shown). Matrix staining did not colocalize with endothelial staining: only part of the matrix proteins was associated with blood vessels (compare Fig. 5a with Figs. 5c and 5e). A layer of matrix proteins was found to surround small groups of tumour cells, suggesting that melanoma cells produced a large part of the matrix proteins themselves. In tumours from the VPF-transfected line I-3, on the other hand, extracellular matrix components were mainly found in the stromal septa: laminin staining was observed throughout the stroma (Fig. 5d), whereas HSPG staining was mainly seen at the interface of the stromal septa with tumour parenchyma (Fig. 5f). Also collagen IV staining was most intense at the boundary between the septa and the tumour nests (not shown). Similar patterns were observed in tumours from other VPF-transfected lines (not shown). Within the tumour cell nodules, a dotted matrix staining was observed in tumours from line I-3 and other VPF-transfected tumours (Figs. 5d and 5f). Only occasionally and locally a fine network of matrix proteins as present in control tumours was observed (see e.g. Fig. 7i). These results suggest that extracellular matrix deposition between melanoma cells is inhibited in the tumours from VPF-transfected lines, either through downregulation of matrix protein synthesis, or through enhanced matrix protein breakdown.

In early tumours from lines E1 and I-3 the typical matrix patterns were already visible. A fine network of laminin and HSPG was present in a small E1 tumour, whereas laminin staining in a small line I-3 tumour was seen almost exclusively in stromal septa (not shown).

Distribution of VPF protein in the xenografts

VPF is thought to exert its activity on endothelial cells, and has been found to accumulate on tumour blood vessels (44). To study the distribution of VPF in the melanoma xenografts sections were stained with a polyclonal anti-VPF antibody. In general low staining intensities were found, especially in control tumours (from lines Mel57, E1, or E2). Diffuse VPF staining throughout the tumours was found in cross-sections of line I-3, I-4, and III-8 tumours, indicative for the fact that VPF is expressed constitutively by these tumours. More intense VPF staining was often seen in the stromal compartments of VPF-transfected tumours (Figs. 6d and 6e) as well as of control tumours (Fig. 6b). VPF staining colocalized, in part, with endothelial cells, confirming the finding that VPF accumulates on tumour blood vessels, but it was also seen on stromal cells that did not stain with the endothelial marker (compare Figs. 6b and 6c). Although especially VPF_{189} has a high affininty for heparin, VPF staining in tumours from the line expressing this variant, III-8, or from other lines, did not colocalize with HSPG staining (not shown).

Hypoxia was suggested to be a cause for the observed upregulation of VPF expression in Mel57 xenografts (33). This hypothesis predicts that higher levels of VPF should be expressed around necrotic areas in such tumours. In some sections of line Mel57, E1, and E2 tumours, staining for VPF was indeed most intense in cells lining necrotic areas (Fig.

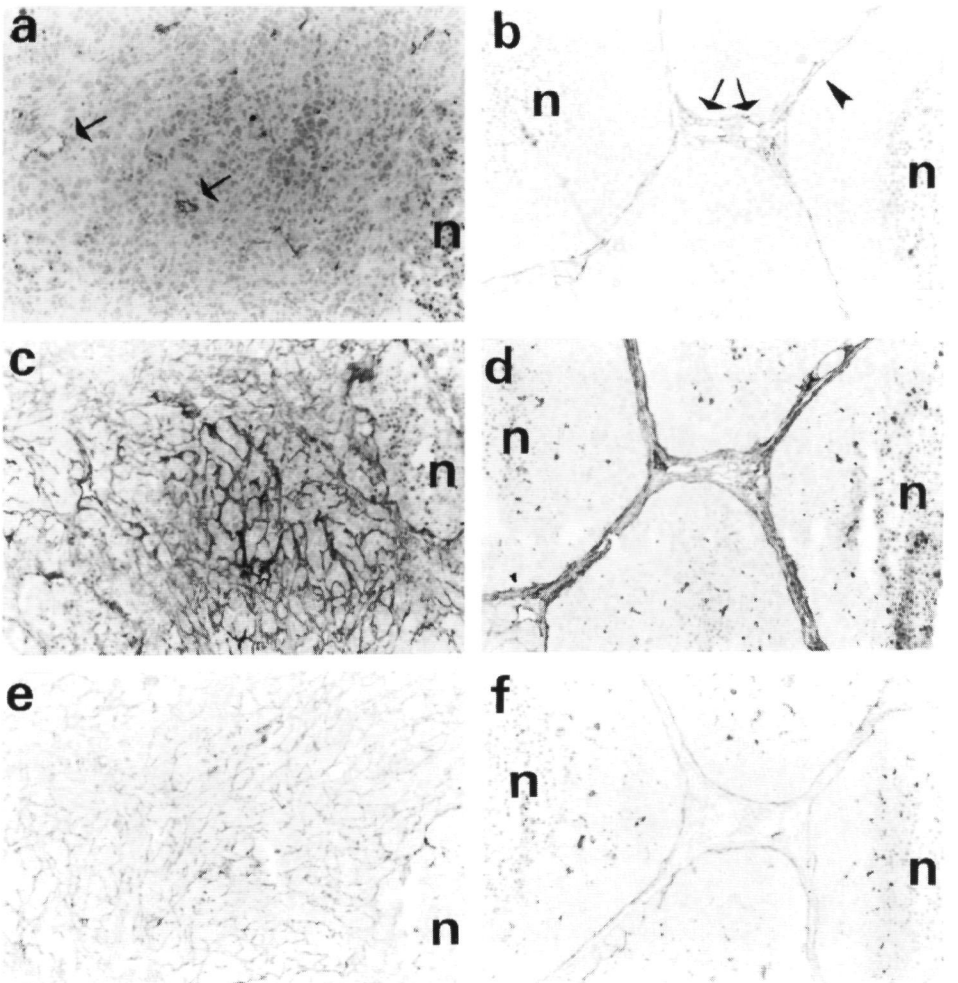


Figure 5. Comparison of endothelial and matrix staining in tumours from transfected melanoma lines. Serial sections of a line E1 tumour (**a, c, e**) and a line I-3 tumour (**b, d, f**) were stained with the endothelial-specific monoclonal (**a, b**), an anti-laminin polyclonal (**c, d**), and an anti-HSPG polyclonal (**e, f**). Necrotic regions are marked "**n**", blood vessels are marked by **arrows**, and a double layer of endothelium in a stromal septum in panel **b** is marked by an **arrowhead**. Note that in the line E1 tumour only a few vessels are visible and that the matrix distribution is dense and chaotic, whereas in the line I-3 tumour both the endothelial staining and the matrix stainings yield the same H-shaped figure formed by the boundaries of several tumour nodules. Magnifications: 80x.

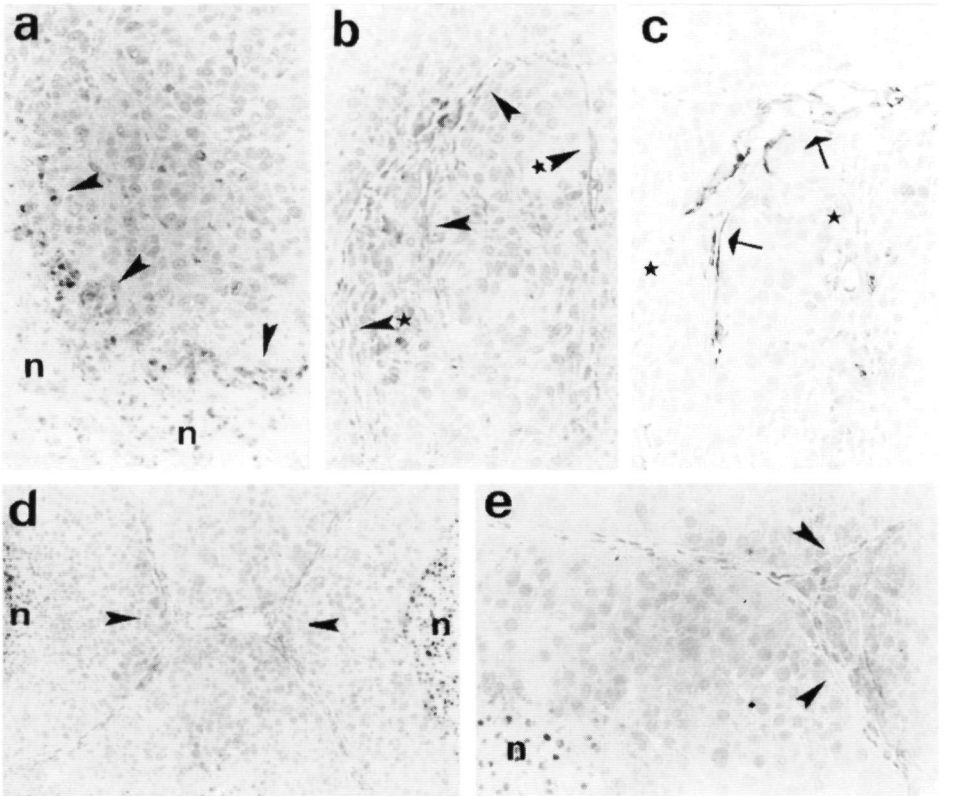


Figure 6. Localization of VPF in tumours from untransfected and transfected melanoma lines. Sections of tumours were stained with a polyclonal anti-VPF/VEGF antibody (**a, b, d, and e**) or with the anti-endothelial antibody (**c**). Tumours were from the following lines: **a:** Mel57, parental line; **b** and **c:** E1, serial sections showing the same region of a small tumour (1.5 x 0.8 mm) stained for VPF and endothelium, respectively; **d:** I-3, serial section to Figs. 5b, 5d, and 5f; **e:** III-8. Necrotic areas are marked "**n**". Delicate VPF staining is faintly visible in black and white reproduction. Therefore, the regions staining for VPF are also marked by **arrowheads**. In panel **c**, endothelial staining is marked by **arrows**. **Asterisks** indicate non-endothelial stromal structures in panel **c** that do stain for VPF in panel **b**. Magnifications: **a-c and e:** 160x; **d:** 80x.

6a), suggesting an influence of hypoxia on the induction of VPF expression in the control tumours. However, there was not always an intense VPF staining seen adjacent to necrosis, while in a very small line E1 tumour without any necrosis VPF staining was already visible in the stroma (Fig. 6b). Therefore, tumour necrosis appears to be neither necessary nor sufficient for the induction of VPF expression, and other mechanisms than hypoxia must be involved in this induction as well.

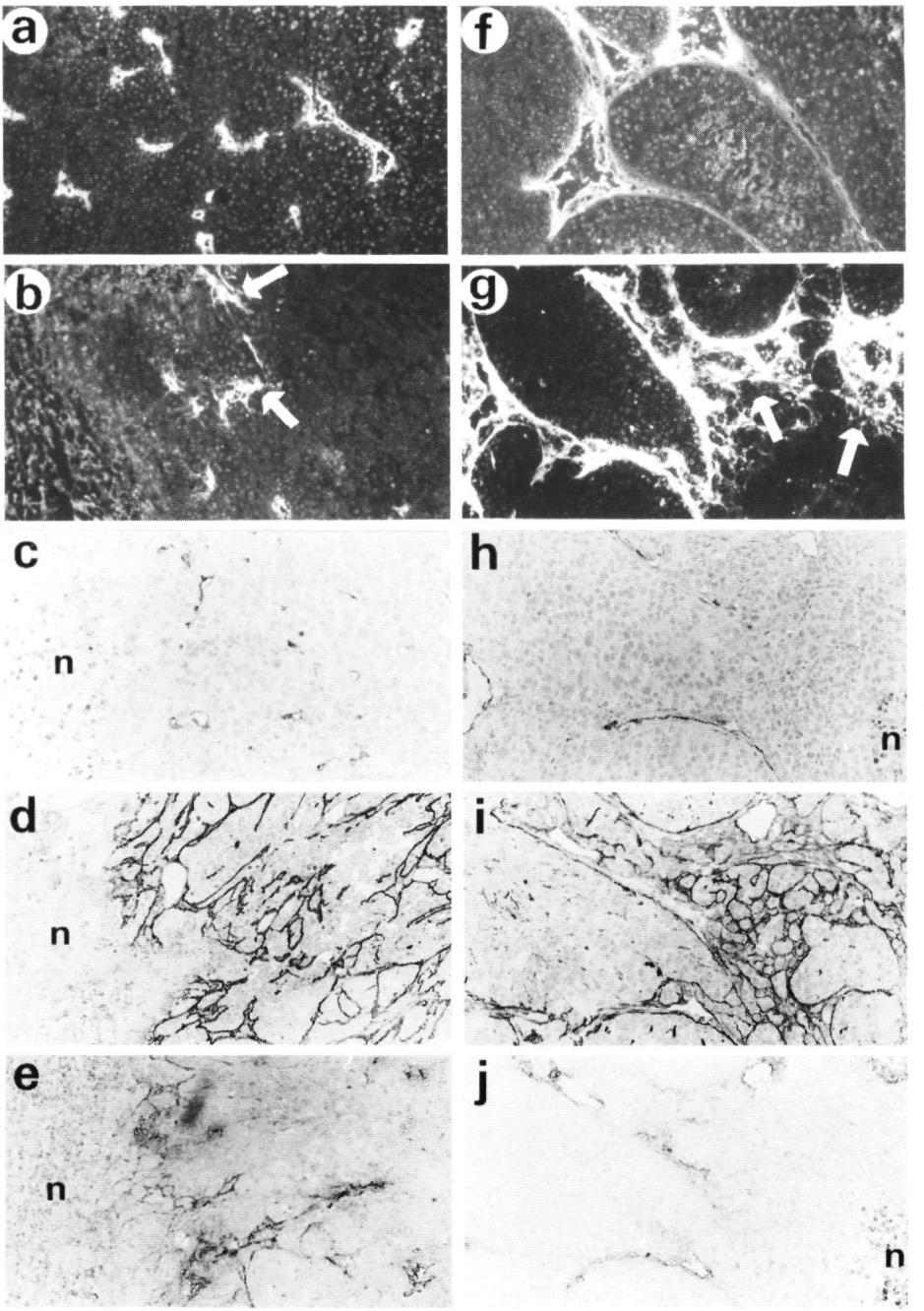
Examination of vascular permeability in the transfectant xenografts

Since VPF is an inducer of vascular permeability (29), the permeability of the blood vessels for proteins may be enhanced in tumours derived from the VPF-transfected lines. This was tested by i.v. injections of fluorescein-conjugated albumin (FITC-BSA), and subsequent examination of the tumours.

In tumours from line Mel57 or from vector-transfected lines, 2 min and 10 min after injection very little extravasation of tracer had occurred: fluorescence was almost excusively seen within the vascular walls (Fig. 7a). After 45 min, however, small amounts of tracer had leaked from the vessels into the tumour mass, visible as short protrusions coming out of the vessels (Fig. 7b). In all VPF-transfected tumours, 2 min after injection, tracer was found not only in the walls of what appeared to be functional blood vessels (Fig. 7f), but part of it was also found in the rest of the stromal septa. These septa, therefore, seemed to contain channels connecting the blood vessels. These channels partly colocalized with endothelial cells, and thus they may be part of the vascular system. Very extensive leakage from the vascular system into the tumour mass was evident 10 min and 45 min after injection, as tracer was not only seen in the stromal compartments rich in endothelial cells, but had also spread through parts of the tumour parenchyma (Fig. 7g). Still, the tracer was retained within parts of the tumour cell mass rich in matrix proteins (Figs. 7g and 7i, right hand side), which apparently formed a fine network of interstitial channels. In the control tumours a similar or even more extensive network of matrix proteins was present (Figs. 5c, 5e, 7d), but tracer leakage was much less intense (see above). The centres of the tumour nodules, poor in matrix proteins, were not reached by the protein tracer (Fig. 7g), in accordance with earlier reports on protein leakage from tumour blood vessels (13,44). These data thus show that in VPF-overproducing tumours protein leaks more extensively from the tumour vessels into the tumour cell compartment than in control tumours, and that the distribution of the extravasated protein in the tumour parenchyma colocalizes with a network of extracellular matrix.

As a consequence of this higher vascular permeability, the formation of an extravascular fibrin matrix may be favoured, facilitating the growth of new blood vessels (32), or directing the pattern in which angiogenesis takes place. Staining for fibrinogen/fibrin in

Figure 7. Leakage of macromolecular tracer from blood vessels and deposition of extravascular fibrin. FITC-labelled BSA was i.v. injected in mice bearing line Mel57 tumours (**a-e**) or line I-4 tumours (**f-j**). Sections shown in panels **a** and **f** are from tumours removed 2 min after injection, panels **b** and **g** show tumours removed 45 min after injection. Tracer was visualized by fluorescence microscopy. Leakage of tracer is indicated by **arrows**. Panels **c-e** are serial sections corresponding with panel **b**; panels **h-j** are serial sections corresponding with panel **g**. **c** and **h:** staining with the anti-endothelial antibody, showing the distribution of blood vessels; **d** and **i:** staining with an anti-HSPG antibody, showing the distribution of matrix proteins; **e** and **j:** staining with an anti-fibrinogen/fibrin antibody, showing the deposition of (extravascular) fibrin. Necrotic areas are marked "**n**". Magnifications: 80x.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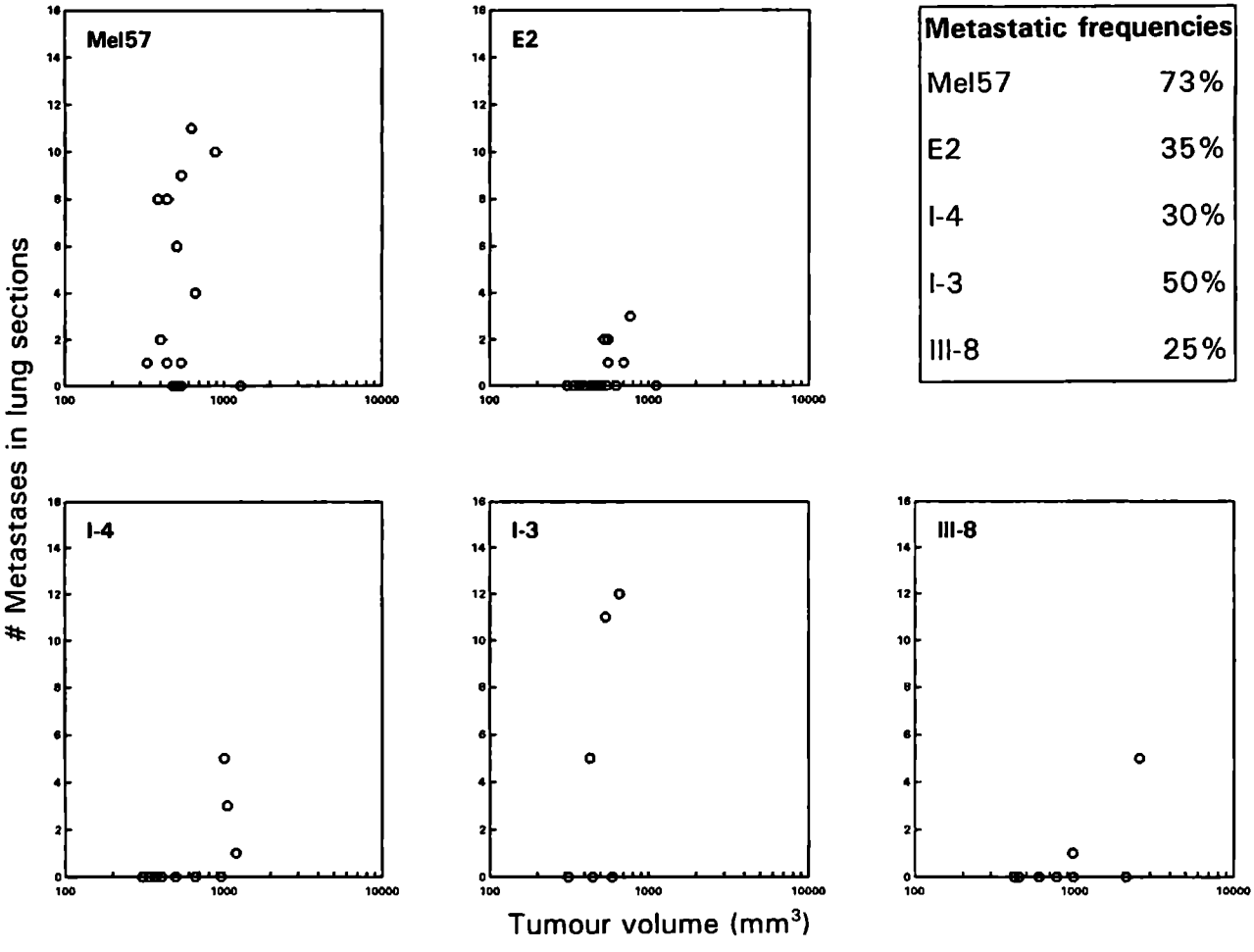
cross-sections of these tumours did not show significant differences in the distribution of fibrin, however. Fibrin staining was most intense in the vascular walls, but some fibrin deposition was also visible as short protrusions from blood vessels in all types of tumours (Figs. 7e and 7j). The network of extravascular fibrin was certainly not more extensive in VPF-overproducing tumours. Apparently, extravasated fibrin was deposited close to the blood vessels, and protein leakage from tumour vessels was not rate-limiting for the development of an extravascular fibrin matrix.

Metastatic properties of the transfectant xenografts

Since tumour angiogenesis is believed to be essential for tumour metastasis (1-3), the altered vascular phenotype caused by a different VPF expression pattern may also change the metastatic potential of the tumours. To test the metastatic properties of the transfectant melanoma xenografts, lines Mel57, E2, I-3, I-4, and III-8 were injected s.c. in 10 or 20 mice each. When the tumours reached a size of between 300 and 2000 mm^3, the mice were sacrificed, and the numbers of lung metastases were determined. These numbers were plotted against the volume of the primary tumour at the moment the mice were sacrificed. In this experiment, the percentage of mice bearing a line Mel57 tumour having one or more metastases (73%), and the number of Mel57 metastases found per mouse, were quite high compared with mice with line E2 tumours, and also with most VPF-transfectant tumours (see Fig. 8). Melanoma line Mel57 is of an earlier passage than all the transfected lines at the time of injection, since the latter lines have all been cloned and selected in hygromycin-containing medium for a prolonged period (see the Materials and Methods section). Therefore, vector-transfected line E2 is probably a better control in this experiment than parental cell line Mel57. Similar frequencies of metastasis were found for lines E2 (35%), I-3 (50%), I-4 (30%), and III-8 (25%). In most cases the numbers of metastases per mouse, if present at all, were rather low. In a few mice carrying line I-3 tumours many metastases were found occurring relatively early in tumour development (tumours between 400 and 700 mm^3, see Fig. 8), but the total number of mice with a line I-3 tumour was very small (6). To allow for thorough statistical analysis, much higher numbers of mice would have to be tested, but at present these data do not support the possibility that the alteration in vascular pattern changes the metastatic potential of the tumours.

Figure 8. Metastatic behaviour of the tumours from Mel57 and transfected lines. The number of metastases counted in two cross-sections of both lungs of an animal was plotted as a function of the tumour volume at the time of obduction. The table in the top right hand corner shows the calculated metastatic frequencies for the different lines.



Metastatic frequencies	
Mel57	73%
E2	35%
I-4	30%
I-3	50%
III-8	25%

Discussion

The aim of this study was to test whether a change from an inducible to a constitutive VPF expression pattern in melanoma cells affects the development of the tumour vascular bed, and if so, whether the level of VPF expression influences the vascular pattern. This question was inspired by the observation that human melanoma lines differed in their pattern of VPF expression. Some lines, like BLM, had a constitutively high level of VPF expression, whereas other melanoma lines with a somewhat different vasculature, like Mel57, had a much lower expression level of VPF in culture, which *in vivo* was upregulated to the same level as that found in line BLM (33). By transfection of line Mel57 several lines with a high basal level of VPF expression were obtained. These lines provided a model to test the influence of the VPF expression pattern *in vivo*.

An intriguing observation using the transfectant lines *in vitro*, was the higher efficiency of translation of the recombinant VPF RNA compared to that of the endogenous VPF mRNA. In the 5' non-coding sequence of the endogenous VPF messenger a GC-rich region is present as well as several AUG and GUG alternative initiation codons (43). Such features have also been found in the 5' non-coding region of the PDGF-B chain transcript, where they have a profound inhibitory effect on the translation efficiency (45,46). The 5' region of the recombinant VPF RNA contained a vector-encoded SV40-derived leader and only a 21 base stretch of the 5' non-coding sequence of the VPF gene. The higher translation efficiency of this recombinant transcript indicates that also in the 5' non-coding region of the VPF mRNA sequences are present that inhibit translation.

When studying and interpreting the differences in biological behaviour induced by the transfection of VPF expression constructs, one should be aware of unexpected genetic changes that can occur during the transfection and selection procedure. Chromosomal integration of the transfected DNA fragment may alter the expression of a gene essential for e.g. metastasis. During selection of transfected cell lines spontaneous mutations may also occur which could influence the biological behaviour of the lines. Finally, spontaneous mutations may occur *in vivo*, and expression of the transfected fragment may be lost because of the absence of hygromycin selection. Mutations may yield a selective advantage *in vivo*, and cause heterogeneity of the tumour cell population. With respect to the vascular patterns, the vascular permeability, and the matrix distribution studied, significant differences were never observed between the parental line Mel57 and the vector-transfected lines on one hand and the various VPF-transfected lines on the other hand. Also morphological heterogeneity within tumours was rarely seen. Therefore, there is no reason to assume that the observed differences in tumour morphology and physiology are caused by unexpected genetic changes; they are much more likely to be caused by the experimentally intended changes in the VPF expression pattern. The difference in metastatic potential observed between tumours from the parental line and from the vector-transfected line E2, however, may be the consequence of an unintended genetic change, and argues caution in interpretating these data.

The lines transfected with VPF expression constructs produced tumours with a strikingly different vascular pattern than the parental line or vector-transfected lines. The vascular pattern seen in tumours from the VPF-transfected lines was, unexpectedly, also very different from that in other melanoma lines constitutively expressing VPF, such as BLM (33, J.R. Westphal et al., unpublished results). This comparison is not quite appropriate, however, as VPF is probably not the only determinant of the vascular phenotype. Furthermore, the VPF expression levels of the transfectant lines are presumably an order of magnitude higher than the expression level of line BLM.

The aberrant vascular phenotype induced by the VPF-transfected lines in the present studies was not dependent on the level of recombinant VPF expression. It should be noted, however, that even in the line with the lowest expression level (I-11), VPF was probably still overexpressed by a factor of 4 to 9 compared with normal Mel57 xenografts. Transfectant lines with even lower expression levels than line I-11 would have been useful, but were difficult to identify by Northern blotting because of their extremely low levels of recombinant VPF RNA.

The vascular pattern in tumours from line III-8, which expresses the heparin-binding VPF_{189} variant, was not significantly different from the pattern in the tumours producing the diffusible variant VPF_{121}. This indicates that this molecular variant is biologically active and that it must, at least in part, be released from the producing cells. Assuming that VPF_{189} is normally retained by heparan sulphate proteoglycan (HSPG) in the extracellular matrix (34), it is important to note that little HSPG appeared to be present in the tumour nodules from all VPF-transfected lines, thus allowing VPF_{189} to diffuse away. Furthermore, proteases like plasmin may be present in the tumour parenchyma which can release a biologically active part of VPF_{189} (34).

It is difficult to give an explanation for the development of the aberrant vascular phenotype in the tumours from VPF-transfected lines. A suitable explanation for the absence of tumour-penetrating vessels in the VPF-overproducing tumours could be the absence of a VPF concentration gradient that would develop in control tumours as a result of hypoxia-induced VPF expression. However, the correlation between tumour necrosis and VPF staining was not as strong as might have been expected if hypoxia were the only inductive factor. Also the fact that different vascular patterns had already evolved in very small tumours from lines E1 and I-3, without any signs of necrosis, makes this hypothesis unlikely.

Varying degrees of extravascular fibrin deposition could influence the patterns of neovascularization in the tumours as well (32). Although the permeability of the tumour vessels for proteins was, as expected, chronically increased in the tumours producing recombinant VPF, it did not lead to a more extensive fibrin network in these tumours. Extravasated fibrinogen may be quickly converted into fibrin, and be deposited close to the blood vessels from which it originated. Furthermore, along with larger amounts of extravasating fibrinogen, also larger amounts of plasminogen may leak from the vessels. This may be activated by plasminogen activators, which are probably expressed in increased amounts by endothelial cells due to the action of VPF (26), and which may also

be expressed by the tumour cells. Plasmin could then remove most of the deposited fibrin. An enhanced activity of proteinases in the tumours from VPF-transfected lines could also explain the low amounts of matrix proteins detected within their tumour cell nests. It is therefore of great interest to investigate in future studies the presence and distribution of specific proteases in these tumours.

Although changing VPF expression in a melanoma line from inducible to constitutive does change the pattern of tumour vascularization qualitatively, the mechanism by which VPF influences this pattern is still unclear. The determination of the vascular pattern is clearly dependent upon a number of parameters, of which VPF expression is one, that are not quite understood. Therefore, further studies on the role of VPF in angiogenesis and tumour vessel function are required.

The idea that the tumour vasculature is important for metastasis, urged us to study the consequences of varying vascular types on the metastatic potential of the tumours. As yet we could not demonstrate significant differences in the metastatic behaviour between the tumours from a vector-transfected line and several VPF-transfected lines. The data are difficult to interpret, however. The parental line Mel57 produced tumours with a higher frequency of metastasis despite the fact that its vascular morphology was indistinguishable from that in tumours from vector-transfected line E2. Mel57 was of a lower passage at the time of injection into mice, and had not undergone the transfection and selection procedures, and therefore may not be a fair control line in these experiments. On the other hand, unexpected events may have genetically altered line E2 (as discussed above). This possibility is hard to verify, but it does undermine the reliability of line E2 as a control in the metastasis experiment.

Initially we expected that overexpression of VPF would increase the number of tumour blood vessels. However, in spite of the dramatic differences in the vascular patterns observed between the control tumours and the VPF-overproducing tumours, the density of functional blood vessels was not obviously altered. Hence, this system may not be a suitable model to test if the number of tumour blood vessels is rate-limiting to the process of metastasis. An intense staining with markers for laminin and proteoglycans in the stromal septa of VPF-overproducing tumours, suggests that these matrix proteins act as a basal membrane, forming a barrier between tumour cells and the highly vascular stroma. This is another complicating factor in this model system.

It would be useful for future experiments if melanoma lines were found which do not upregulate VPF expression *in vivo*. Melanoma lines forming very slowly growing tumours in nude mice do exist, but their VPF expression *in vivo* was not studied, just because of this practical disadvantage. However, such tumours might have low VPF expression levels, which would make their cell lines attractive candidates for transfections with VPF expression constructs. Such an approach might improve the vascularization of the resulting tumours, yielding a model very suitable for studying the influence of tumour vascularization on the process of metastasis.

Acknowledgements

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Chapter 5

Covalent Dimerization of Vascular Permeability Factor is Essential for its Biological Activity - Evidence from Cys to Ser Mutations

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Vascular permeability factor, or vascular endothelial growth factor (VPF/VEGF), is an important factor in the regulation of vascular growth and vascular permeability. VPF is a secreted, dimeric protein and has eight cysteine residues conserved with platelet-derived growth factor (PDGF). To study the role of some of these cysteine residues in maintaining the structure and function of VPF, we replaced the codons for the 2nd, 3rd, 4th, and 5th cysteine by serine codons, and expressed the mutant proteins in a mammalian expression system. Cysteine residues 2 and 4 in VPF were found to be directly involved in anti-parallel interchain disulfide bonds, as in PDGF. VPF mutants lacking one of these cysteines were severely impaired in their S-linked dimerization, whereas upon coexpression of both mutants the ability to form dimers was restored. The VPF mutants lacking cysteine residue 2 or 4 also competed poorly for receptor binding of labelled VPF and had low biological activity, but these defects were also complemented by coexpressing the two mutants, indicating that for efficient receptor binding and activation VPF needs to be a covalent dimer, unlike PDGF-BB. Furthermore, cysteine residue 5 was found to be essential for VPF dimerization and activity, whereas the mutant lacking cysteine residue 3 was only mildly affected in its ability to dimerize and had partial biological activity.

Introduction

Vascular permeability factor (VPF) was first discovered as a protein factor that increases vascular permeability (1), but upon cloning of its gene it appeared to be identical to vascular endothelial growth factor (VEGF), an inducer of endothelial cell proliferation and angiogenesis (2,3). More recently, other activities of VPF/VEGF have been described, such as its ability to induce the expression of tissue factor, plasminogen activators, plasminogen activator inhibitor-1, and interstitial collagenase by endothelial cells (4-6), and its ability to attract and activate monocytes (4,7). VPF has an N-linked glycosylation site (2,3) but is often found as a mixture of glycosylated and non-glycosylated proteins (8,9). Glycosylation does not appear to be essential for biological activity of VPF, but does affect the efficiency of cellular secretion (8).

Two different receptors for VPF have been characterized and cloned, which appear to be expressed only by endothelial cells and haemopoietic cells (10,11). Apart from its expression during physiological processes (12-16), VPF is thought to be an essential factor in tumour progression (17-19). Therefore it is useful to investigate the structure of VPF, as this may help in the design of VPF inhibitors.

Due to alternative splicing of the VPF messenger, various molecular variants of VPF exist with different isoelectric points, affinities for heparin, and cysteine contents. The smallest variant VPF_{121} contains 9 cysteine residues, while larger variants contain 16 cysteine residues (9). VPF has been classified as a member of a growth factor family to which also the platelet-derived growth factors (PDGF) belong. VPF has an amino acid homology of 15-18% with the PDGF-A and -B chain, but most notable is the perfect conservation of eight cysteine residues between these proteins (2). In the PDGF-A and -B chain, two of the eight cysteine residues are directly involved in dimerization: the 2^{nd} cysteine of one subunit forms an intermolecular disulfide bridge with the 4^{th} cysteine of the other, and vice versa (20,21). Mutations of either cysteine 2 or cysteine 4 to a serine residue abolished S-linked dimerization of PDGF, but a heterodimer could be formed by coexpression of both mutants (20). The Cys to Ser mutants of the PDGF-B chain still bound to their receptor and had agonist activity. Recently, evidence was presented that these mutants still form dimers which are kept together by non-covalent interactions (22). The monomeric Cys2 to Ser and Cys4 to Ser PDGF-A chain mutants, on the other hand, did not bind to their receptor, but heterodimers formed by these mutants had recovered receptor binding and biological activity (20). The other six cysteine residues in the PDGF-B chain are involved in intra-subunit disulfide bridges: in the crystal structure Cys1 is linked to Cys6, Cys3 to Cys7 and Cys5 to Cys8 (21). These six cysteine residues are conserved in nerve growth factor (NGF) and transforming growth factor-ß2 (TGF-ß2) as well, which also share the same arrangement of intramolecular cystine bridges, called "cystine knot", with PDGF-BB (23-25).

Replacement of cysteine residues to serine residues in v-*sis*, a homologue of the PDGF-B chain, revealed that cysteines 1, 3, 6, and 7 are essential for biological activity, whereas mutants lacking cysteines 2, 4, 5, and 8 still have transforming activity (26,27).

All eight Cys to Ser mutants were impaired in their ability to form S-linked dimers, however, suggesting that also the intramolecular disulfide bridges may be essential for dimerization, by maintaining a proper subunit structure.

In VPF, as stated above, eight of the cysteine residues are conserved with PDGF. Therefore, it is tempting to assume that VPF has the same arrangement of intramolecular and intermolecular disufide bridges as PDGF. As yet no study has been reported in which this hypothesis is tested. Furthermore, it is unknown if VPF dimerization is essential for receptor binding and activation. In this paper we describe the expression of VPF mutants with the 2nd, 3rd, 4th or 5th cysteine residue replaced by a serine residue. Cysteines 2 and 4 appear to be involved in intermolecular disulfide bridges analogous to the situation in PDGF. In contrast with earlier findings with v-*sis*, cysteine 5 was also essential for VPF dimerization and activity, whereas dimer formation and biological activity were only slightly affected by the mutation in cysteine 3. Our data further indicate that, in contrast to PDGF-BB, covalent dimerization of VPF is essential for effective receptor binding and biological activity.

Materials and Methods

Production and expression of VPF cysteine to serine mutants

A cDNA clone containing the protein coding region of VPF_{165} was obtained by RT-PCR on RNA from phorbol 12-myristate 13-acetate (PMA)-stimulated cell line U937 (2). The PCR product was cloned into vector pBluescript II KS+ (Stratagene, San Diego, CA, USA) from which single stranded antisense template was prepared. The protocol for site-directed mutagenesis was adapted from (28). 20- or 21-mer primers were designed which corresponded to the 2nd, 3rd, 4th, or 5th cysteine (amino acids 51, 57, 60, and 61, respectively) and surrounding amino acids, but carrying a G to C change at the second cysteine codon position (see also Fig. 1). The phosphorylated primers were annealed with the template and a second DNA strand was synthesized using DNA polymerase Klenow fragment (Gibco BRL, Paisley, UK). VPF mutant clones were obtained by transformation to *E. coli* strain BMH71-18 mut S (29) and subsequently to strain XL-1 Blue (Stratagene, San Diego, CA, USA), and were identified by sequencing. The wild type and the four mutant VPF sequences were recloned as *SalI/KpnI* fragments to vector pSPTBM20 (Boehringer Mannheim, Germany), and then as *SalI/NotI* fragments to the expression vector pBPV (Pharmacia LKB, Woerden, The Netherlands) by ligation into the *XhoI* (5') and *NotI* (3') sites. The plasmids were transfected into mouse C127I cells (ATCC, Rockville, MD, USA) using DOTAP (Boehringer Mannheim, Germany) as transfection reagent. Transfected cells were first cultured for 6 weeks in Dulbecco's Modified Eagle's Medium (DMEM, Flow laboratories, Irvine, UK) supplemented with 10% fetal calf serum (FCS, Gibco BRL, Paisley, UK), L-glutamine, penicillin and streptomycin, to allow for enrichment of cells transformed by the BPV sequence (30). After this period,

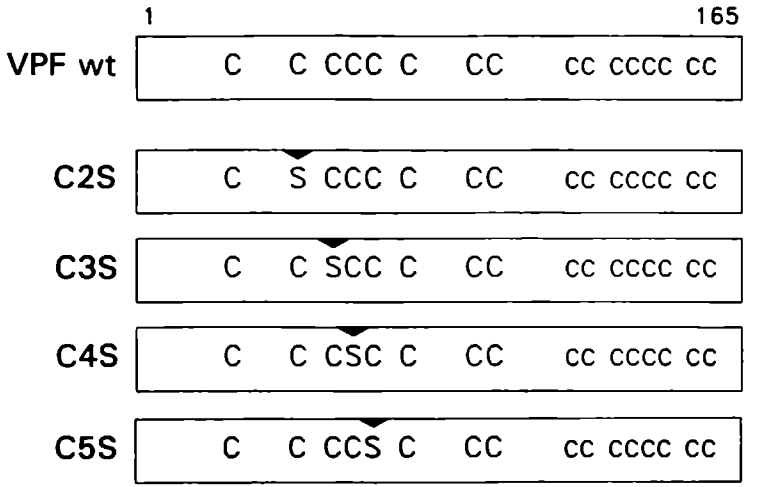

Figure 1. Schematic illustration of cysteine residues in VPF_{165} and the serine substitutions in VPF mutants. The top bar represents mature wild type VPF_{165} from the N-terminus (**1**) to the C-terminus (**165**). The eight large "**C**"s represent the cysteine residues shared with PDGF. In VPF mutants C2S, C3S, C4S, and C5S one of these cysteine residues is substituted for a serine residue (**S**, **arrowhead**).

VPF or VPF mutants were harvested by placing cells in DMEM without serum for 24 hour periods twice a week. The recombinant proteins continued to be expressed for at least 6 weeks. To double-check that the transfected lines contained the appropriate constructs, DNA was isolated from the transfected cells, amplified by PCR, and hybridized to the original mutant primers.

Production of polyclonal antiserum against VPF

For large scale expression of VPF in a bacterial system the last two arginine codons in the VPF_{165} cDNA were changed into a *BamHI* site by PCR, and a 0.5 kb *NcoI*(blunt)/*BamHI* fragment was recloned to the *BamHI*(blunt)/*BglII*-digested vector pQE16 (QIAGEN, Westburg, Leusden, The Netherlands). The resulting plasmid, pQE-NcoVPFII, was transformed into *E. coli* strain M15pREP4 (QIAGEN). The recombinant protein expressed by these cells consisted of the VPF amino acids 3-163, plus 4 additional amino acids at the N-terminus and a Gly-Ser-$(His)_6$ sequence at the C-terminus. IPTG-induced bacteria were lysed in 6 M guanidinium chloride, and the recombinant protein was purified on a Ni-NTA column (QIAGEN) by elution with 8M urea and a descending pH gradient. The purified protein was acetone precipitated, dissolved in phosphate-buffered saline (PBS) with 0.1% SDS, and injected into a rabbit 3 times to obtain antiserum against VPF. To minimize non-specific staining of high molecular mass serum-

derived bands on Western blots, the antiserum was blocked before use with 3 volumes of heat-denatured FCS.

Determination of protein concentrations

Before analysis by electrophoresis, VPF mutants were precipitated from samples of serum free conditioned media by addition of 2 volumes of acetone. To determine the concentrations of VPF mutants in the conditioned media the precipitates were electrophoresed on SDS-PAGE under reducing conditions, together with a range of known amounts of commercially obtained recombinant human VPF/VEGF (Peprotech, Sanvertech, Breda, The Netherlands). Gels were electroblotted to nitrocellulose membranes, which were incubated in FCS-blocked and 250-fold diluted antiserum against VPF and stained with the chemiluminescent substrate AMPPD (Tropix, Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. Intensities of bands were quantitated by laser scanning.

Analysis of VPF mutant dimerization

To determine the ability of VPF mutants to form S-linked dimers, precipitates from conditioned medium were electrophoresed on SDS-PAGE without prior reduction. As the antiserum stained fully reduced VPF stronger than non-reduced VPF, gels were incubated before electroblotting in electrophoresis running buffer with 5% ß-mercaptoethanol for 10 min at 100°C. Immunodetection of VPF was performed as described above. To study the ability of mutants to form non-covalently linked dimers, proteins were trapped in the dimeric state by adding the cross-linking agent disuccinimidyl suberate (DSS; Pierce, Oud Beijerland, The Netherlands) to conditioned media to a final concentration of 2.5 mM. After 90 min at 37°C the reactions were stopped by addition of glycine to 100 mM and a further incubation of 10 min at 37°C. Precipitations, SDS-PAGE under reducing conditions, blotting, and immune detection were performed as described above.

Biological assays

For biological assays crude centrifuged conditioned media from C127I cells were used, in which VPF concentrations had been determined by Western blotting (see above). Samples containing VPF or VPF mutants were diluted to the appropriate volumes with conditioned medium from C127I cells transfected with pBPV vector DNA only (control supernatant, or pBPV supernatant) to keep protein backgrounds as constant as possible. Procoagulant assays, measuring tissue factor expression, were performed with human umbilical vein endothelial cells (HUVEC), essentially as described elsewhere (4,31). To 0.75 ml of assay medium 0.25 ml samples of conditioned medium containing VPF or VPF mutants were added, and after 5 h tissue factor amounts were determined. Mitotic assays were performed on microvascular endothelial cells (MVEC) from human foreskin

(provided by Dr. V. van Hinsbergh, Leiden, The Netherlands). These cells were cultured as described in (32). For assays they were plated in 24-well tissue culture plates coated with gelatin. $1.5x10^4$ cells were seeded per well in 0.9 ml M199 (Flow laboratories, Irvine UK) supplemented with 5% newborn calf serum (Gibco BRL, Paisley, UK), 5% human serum, 20 mM HEPES, pH 7.3 (ICN, Zoetermeer, The Netherlands), L-glutamine, penicillin and streptomycin. After 24 h 100 μl samples of VPF (mutants) were added, and another 48 h later 0.5 μCi [^{3}H]thymidine (ICN, Zoetermeer, The Netherlands) was added to each well. Cells were incubated for 6 h in 7.5% CO_2 at 37°C, washed 3 times with PBS, fixed in methanol, and lysed in 0.2 N NaOH. [^{3}H]Thymidine incorporation was measured by scintillation counting. Miles-type vascular permeability assays were performed essentially as in (1,33). In short, anaesthesized and shaved albino guinea pigs were i.v. injected with Evans Blue, i.d. injected with 100 μl samples of VPF (mutants), and sacrificed 15 min later. The extravasated dye was extracted from skin sections in formamide at 45°C and quantitated spectrophotometrically at 620 nm.

Competition for ^{125}I-VPF receptor binding

2 μg of purified VPF/VEGF (Peprotech, Sanvertech, Breda, The Netherlands), dissolved in 10 μl PBS, was labelled with 0.5 mCi [^{125}I]NaI (Amersham, Amersham, UK) using the Iodo-Beads protocol (Pierce, Oud Beijerland, The Netherlands), and was recovered from a Sephadex G50 column equilibrated in PBS with 0.1% BSA. Specific activity was $3.9x10^7$ cpm/μg protein. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described elsewhere (34,31). For receptor binding experiments these cells were cultured to near confluence in 24-well plates, washed twice with binding buffer (DMEM with 25 mM HEPES, pH 7.0, 0.1% BSA, and 0.15% gelatin), and placed in 0.25 ml binding buffer (C127I cell-conditioned medium instead of fresh DMEM) containing $1.3x10^5$ cpm ^{125}I-VPF and varying concentrations of unlabelled VPF or VPF mutants. For positive and negative controls commercially obtained VPF/VEGF and recombinant bFGF (a gift from Scios Inc, Mountain View, CA) were added. Incubations were for 90 min at 20°C and were followed by three washes in PBS with 0.1% BSA, lysis in 0.25 ml 1% Triton X-100, 20 mM Tris/HCl, pH 7.5, and γ-counting.

Results

Expression of VPF mutants

A prerequisite for the expression of recombinant VPF in a mammalian expression system is that the host cells do not produce VPF themselves. After screening a number of expression systems we found that mouse C127I cells meet this criterion. These cells, even when transformed by BPV sequences in the vector, did not contain detectable amounts of

VPF mRNA, and did not produce detectable VPF-like activity (not shown) or VPF protein (Fig. 2).

C127I cells were transfected with pBPV vector constructs, which ensure stable episomal maintenance, containing VPF_{165} wild-type DNA or mutations thereof with one of the cysteine codons 2, 3, 4, or 5 substituted for a serine codon (see Fig. 1). Transformed cells secreted VPF protein into the medium for a prolonged period. Concentrations in serum-free conditioned media were between 0.1 and 0.6 μg/ml, as determined by Western blotting under reducing conditions followed by staining with a polyclonal antiserum against VPF (Fig. 2). Wild type VPF and all VPF mutants were apparent as a band of 25 kD, which is the expected size of glycosylated VPF_{165} (9). The VPF used as concentration standard was *E. coli*-produced and non-glycosylated, and therefore migrated faster on these gels (Fig. 2).

To test if conditioned media from pBPV-transformed C127I cells influenced the biological activity of VPF in various assays, the activity of different concentrations of commercially obtained VPF/VEGF diluted in serum-free medium with 0.1% BSA, was compared with the activity after dilution in supernatant from vector-transfected C127I cells. The dose-response curves did not differ significantly, hence C127I cell-conditioned

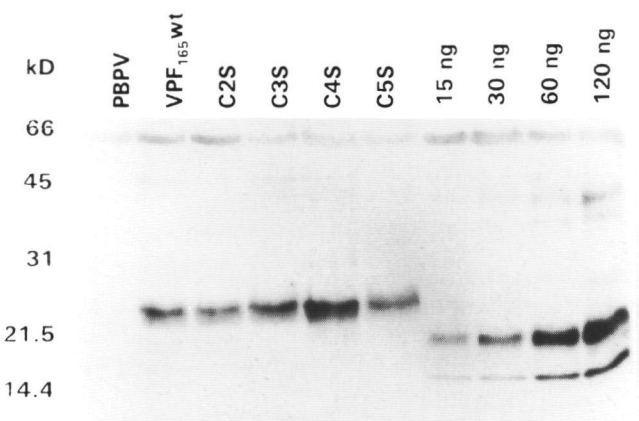

Figure 2. Expression of VPF mutants. Proteins were precipitated from 0.2 ml conditioned media of transfected C127I cells and electrophoresed under reducing conditions. An electroblot of this gel was stained with a polyclonal antiserum against human VPF. **pBPV:** conditioned medium of pBPV (vector)-transfected cells. Concentrations of VPF wild type and Cys to Ser mutants of VPF were determined by comparing band intensities with a dilution range of commercially obtained VPF/VEGF (four right-most lanes), which was expressed in *E. coli*, and therefore non-glycosylated and of a smaller apparent molecular mass (apparently containing a product of an even smaller size as well). Positions of molecular mass markers are indicated on the left. Note that remaining traces of albumin from fetal calf serum in conditioned media, and albumin added to commercially obtained VPF/VEGF as a carrier protein, is weakly stained as a band of 66 kD.

medium did not influence the biological activity of VPF (data not shown). There were also no significant differences observed between the activities of commercially obtained VPF/VEGF and C127I cell-derived VPF (not shown). Thus for the present experiments there was no necessity to purify VPF or its mutants: in the experiments reported below unpurified conditioned media were used.

Dimerization of VPF mutants

The capacity of the cysteine to serine mutants of VPF to form S-linked dimers was tested by SDS-PAGE under non-reducing conditions and subsequent staining of blots with anti-VPF polyclonal antiserum. Wild type VPF_{165} was found as a band of 46 kD, which is the expected molecular mass of the dimeric VPF protein (Fig. 3). All four mutants were more or less impaired in their ability to form S-linked dimers. Mutants in cysteine 2 or 5 (C2S and C5S) were almost completely monomeric, whereas mutants in cysteine 3 or 4 (C3S and C4S) consisted in part of dimers (50% and 20% dimers, respectively). Cysteines 2, 3, 4, and 5 are obviously all important, directly or indirectly, for VPF dimerization.

If dimerization requires interchain disulfide bridges between two different cysteines, then complementation of the dimerization defect should take place upon coexpression of the two mutants with the respective cysteines mutated. Therefore all possible pairs of

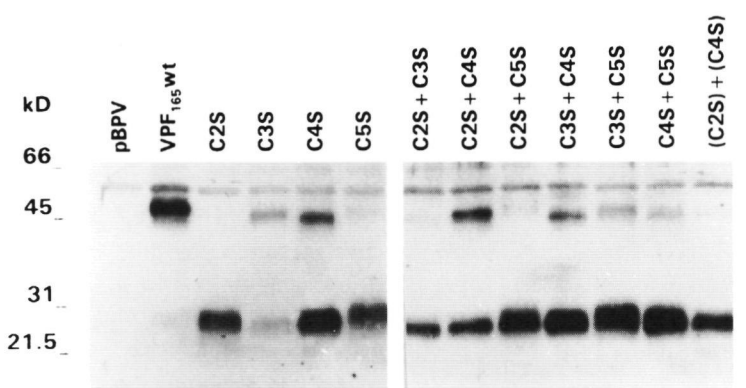

Figure 3. S-linked dimerization of VPF mutants. Proteins precipitated from 0.25 ml conditioned media of transfected C127I cells were electrophoresed under non-reducing conditions, electroblotted and stained with a polyclonal antiserum against VPF. **Left panel:** conditioned medium of pBPV (vector)-transfected cells; VPF wild type; and single Cys to Ser mutants of VPF. **Right panel:** double transfections with two mutants; and **(C2S)+(C4S):** a mix of conditioned media containing mutants C2S and C4S, allowed to interact for 30 min at 20°C before precipitation. Positions of molecular mass markers are indicated on the left. Monomeric VPF is found at approximately 25 kD, whereas dimeric VPF is apparent as a band of 46 kD. Note that a weak albumin stain is visible at the top of the gel.

mutants were cotransfected, and the resulting VPF was tested for dimer formation. As can be seen in Fig. 3 (right panel) only cotransfection of mutants C2S and C4S led to a higher degree of dimerization than found for each of the mutants expressed singly: about 50% of this VPF was dimeric, which was significantly more than the 0-20% dimerization found with the individual mutants. This indicates that the 2nd cysteine residue in one subunit forms an interchain disulfide bond with the 4th cysteine residue in the other subunit, as is the case in PDGF. Nevertheless, VPF mutant C4S is capable of some dimer formation by itself, which cannot be explained by the PDGF dimerization model. Apparently an alternative way of dimerizing is possible for this mutant. Complementation between mutants C2S and C4S was not complete, which may be explained by the fact that the plasmids encoding the mutants C2S and C4S were not present in equal amounts in the transfected cells; in fact the ratio of C2S/C4S sequences 12 weeks after transfection was about 5:2 (data not shown). Mixing of the C2S and the C4S mutants *in vitro* did not lead to extra dimer formation (Fig. 3, right-most lane), suggesting that the disulfide bridge between cysteines 2 and 4 has to be formed intracellularly.

The decreased dimerization level of mutants C3S and C5S could not be complemented by cotransfecting them with other mutants. These cysteine residues may be involved in intramolecular folding, as in PDGF. Mutating these cysteines could lead to changes in subunit folding that are unfavourable for dimerization (C3S) or even abolish dimerization (C5S). However, from the present data it cannot be excluded that cysteine residues 3 and/or 5 are involved in interchain disulfide bridges.

Mutations in the interchain cysteines could still allow non-covalent dimerization. To trap non-covalently associated VPF mutants in the dimeric state they were incubated with the chemical cross-linking agent disuccinimidyl suberate (DSS). This treatment almost completely cross-linked wild type VPF, but also a considerable fraction of mutants C2S, C3S, and C4S was cross-linked by DSS (Fig. 4). The amounts of dimers found after cross-linking of these mutants (65%, 65%, and 40%, respectively) were higher than the amounts of S-linked dimers, especially for mutant C2S (Fig. 3), suggesting that in addition to S-linked dimerization, some non-covalent interaction does indeed take place. Mutant C5S was only 20% dimeric after DSS treatment, which is a similar percentage as found for wild type VPF completely reduced by DTT (18%), thus probably representing non-specific background. The subunits of mutant C5S apparently have a disturbed dimer interface. Heparin had no influence on non-covalent dimerization, since the same amounts of cross-linked dimer were found for all mutants after DSS treatment in the presence of 100 μg/ml heparin (not shown).

Biological activity of VPF mutants

VPF activity can be measured in a number of different biological assays. We used an endothelial cell mitotic assay, a Miles vascular permeability assay, and a procoagulant assay to determine the relative activities of the VPF mutants. In the procoagulant assay, measuring tissue factor expression by endothelial cells, wild type VPF had its maximum

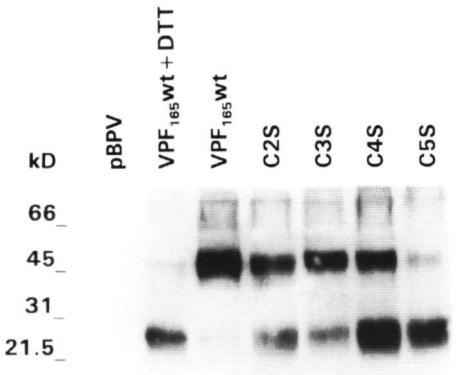

Figure 4. Non-covalent dimerization of VPF mutants. Proteins in conditioned media were chemically cross-linked by DSS, precipitated, electrophoresed under reducing conditions, electroblotted and stained with a polyclonal antiserum against VPF. **pBPV:** Conditioned medium of pBPV (vector)-transfected cells; **VPF_{165}wt + DTT:** wild type VPF after reduction (10 min in 25 mM DTT at 50°C); **VPF_{165}wt:** wild type VPF; **four right-most lanes:** Cys to Ser mutants of VPF. Positions of molecular mass markers are indicated on the left. VPF monomers migrate at 25 kD, cross-linked dimers at 46 kD.

inducing effect at about 25 ng/ml (not shown). VPF mutants were also tested at this concentration, at which mutants C2S, C4S and C5S did not have significant activity, while the partially dimeric mutant C3S was clearly active (Fig. 5). Cotransfections of VPF mutants generally yielded inactive VPF as well, although after cotransfections with mutant C3S partial activity could be observed, as expected from the activity of mutant C3S alone (not shown). The heterodimer formed after cotransfection of mutants C2S and C4S, however, did have full biological activity, which was not seen if VPF mutants C2S and C4S were mixed *in vitro* (Fig. 5, two right-most bars). In the mitotic assay and in the vascular permeability assay similar results were obtained: at concentrations that were just maximal for wild type VPF (5 ng/ml and 20 ng/injection, respectively) significant activity was only seen with VPF mutant C3S and with the heterodimer of mutants C2S and C4S (not shown, but see also Figs. 6 and 7).

These results suggest that S-linked dimer formation of VPF is a prerequisite for biological activity, and that one interchain disulfide bridge from a Cys2 to a Cys4 suffices for full biological activity. One would expect, however, the VPF mutant C4S to be partially active as well, as about 20% of this protein is dimeric. Therefore the VPF mutants were tested for biological activity over a concentration range including higher concentrations. In the mitotic assay, as shown in Fig. 6, mutants C2S and C4S complemented each other when coexpressed, yielding an activity that was comparable with that of VPF wild type, while for mutant C3S about 4-fold higher concentrations had to be added to reach the same effect. VPF mutants C2S and C4S individually showed only little activity at high concentrations, thus the partial S-linked dimerization of mutant C4S does not contribute to its activity in this assay. Mutant C5S gave no evidence of activity in this assay at all. In the Miles vascular permeability assay (Fig. 7) VPF produced upon coexpression of mutants C2S and C4S also had an activity equivalent to that of the wild type protein. Again, mutant C3S required somewhat higher concentrations

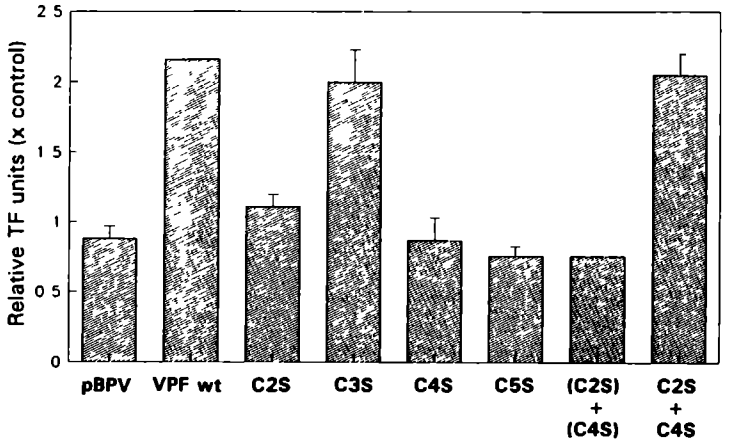

Figure 5. Procoagulant assay. Expression of tissue factor activity by endothelial cells was measured after addition of 25 ng/ml VPF (mutants), the concentration which was maximal for wild type VPF in this assay. **pBPV:** conditioned medium of pBPV (vector)-transfected cells; **(C2S)+(C4S):** VPF mutants C2S and C4S mixed before addition to endothelial cells; **C2S+C4S:** mutants C2S and C4S coexpressed. For details see "Materials and Methods". The procoagulant activity measured after addition of serum-free medium was arbitrarily defined 1. The values presented are the means and standard deviations (SDs) of duplicate measurements.

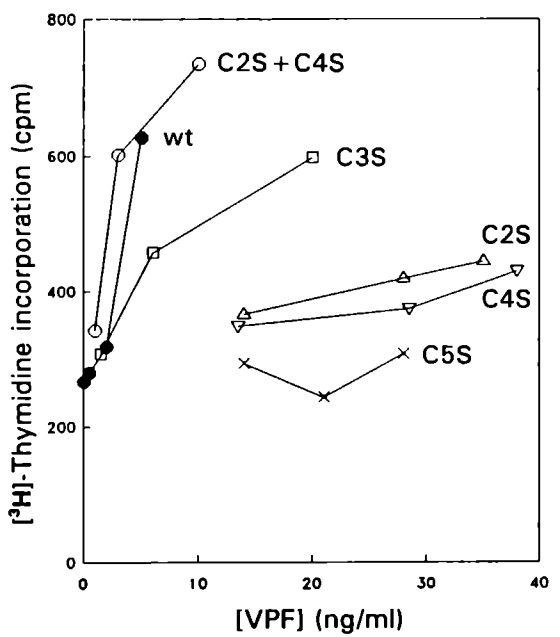

Figure 6. Thymidine incorporation assay. The effect of wild type VPF (●); coexpressed VPF mutants C2S and C4S (○); mutant C2S (▵); mutant C3S (□); mutant C4S (▿); and mutant C5S (x) on DNA synthesis in endothelial cells. See "Materials and Methods" for further details. Values are the means of triplicate measurements. Error bars were omitted for clarity; SDs were between 5-35%.

(about 3-fold) to reach the same activity as wild type VPF. Of mutant C4S a 5-10-fold higher dose was needed to obtain an effect equal to that of the wild type protein, whereas mutants C2S and C5S only started to show some activity at the highest concentrations used. Interestingly, in the permeability assay mutant C4S is reproducibly more active than mutant C2S, in contrast to the mitotic assay, in which mutants C2S and C4S both have comparable (low) activities. For practical reasons, we could not test VPF mutants at high concentrations in the procoagulant assay.

To test whether any of the VPF mutants had antagonist activity, wild type VPF was mixed with increasing concentrations of the biologically poorly active mutants, and the mixture was tested in biological assays. Thus 3 ng/ml of wild type VPF was tested along with 5-40 ng/ml of mutants C2S, C4S, and C5S in the mitotic assay, and 20 ng of wild type VPF was tested together with 50-160 ng of mutants C2S and C5S in the permeability assay. In no case did any mutant inhibit the activity of the wild type protein. On the contrary, at the highest concentrations of some mutants the biological response was slightly enhanced (results not shown).

Competition for receptor binding by VPF mutants

The inability of the poorly active VPF mutants to inhibit the biological activity of wild type VPF, suggests that these mutants do not bind to VPF receptors. This was verified by

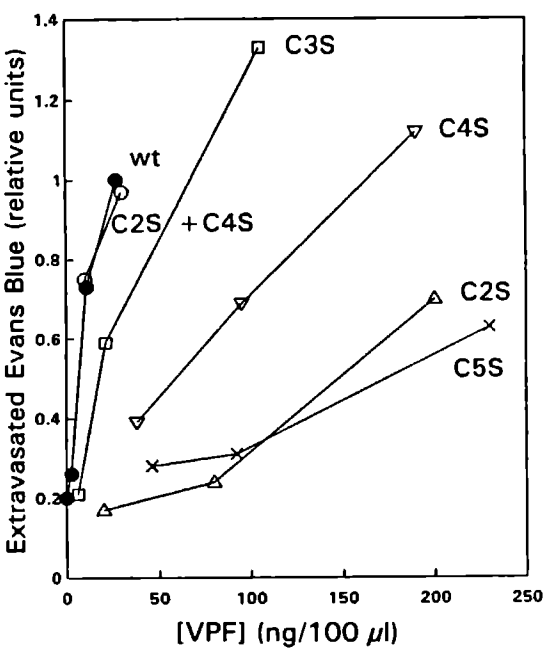

Figure 7. Miles-type vascular permeability assay. The effect of wild type VPF (●); coexpressed VPF mutants C2S and C4S (○); mutant C2S (△); mutant C3S (□); mutant C4S (▽); and mutant C5S (x) on vascular leakage in guinea pig skin. For further details see "Materials and Methods". This graph is a composite of data obtained using two animals. In each of the animals negative and positive control samples (pBPV supernatant and 20 ng wild type VPF) yielded a five-fold difference in Evans Blue leakage (3.35-16.7 µg for animal 1 and 1.58-7.5 µg for animal 2). All measured values were then converted into arbitrary units of leakage, whereby the effect of 20 ng wild type VPF was designated as 1.

measuring the capacity of the VPF mutants to compete with ^{125}I-labelled VPF for receptor binding. Binding of labelled VPF to endothelial cells was most effectively inhibited by wild type VPF (Fig. 8), requiring about 5 ng for 50% inhibition. Mutants C2S and C4S also inhibited receptor binding of labelled VPF, but much less efficiently than the heterodimer formed by these two mutants, which competed for receptor binding only a little less efficiently than wild type VPF, as did mutant C3S. No inhibition of VPF receptor binding was evident for VPF mutant C5S, and for an other heparin-binding growth factor: basic fibroblast growth factor (bFGF) at the concentrations used. In the presence of 100 μg/ml heparin the competition patterns were similar to those without heparin, although total binding of labelled VPF to endothelial cells was about 10-20% lower in the presence of heparin (not shown).

Discussion

If the structure of VPF is indeed homologous to that of PDGF, cysteines 2 and 4 should be involved in interchain disulfide bridges. The data presented here confirm the PDGF model for VPF: mutants lacking cysteine 2 or 4 were impaired in their ability to dimerize, but crucially, mutants C2S and C4S complemented each other's defect when coexpressed. VPF dimerization thus is dependent on the formation of disulfide bridges between the 2nd cysteine of one subunit and the 4th cysteine of the other, and vice versa, leading to a doubly linked dimer which will have an antiparallel arrangement of the two subunits. For PDGF this crosswise orientation of intermolecular disulfide bridges was originally deduced from mutational experiments similar to those presented here, and the model was later confirmed by crystallography (20,21).

Unexpectedly, mutant C4S consisted of about 20% dimers, which cannot be explained by the dimerization model for PDGF. The cell line that expressed this mutant did not contain other plasmids than the one with the C4S mutant sequence, therefore this low amount of dimers is not caused by a contamination but is a feature of the mutant itself. A PDGF-A chain mutant with its 4th cysteine changed to a serine did not show any signs of S-linked dimerization (20); in v-*sis*, on the other hand, both for the Cys2 to Ser mutant as for the Cys4 to Ser mutant there was evidence of some dimer formation (27). The C4S mutant of VPF must utilize an alternative way to dimerize, possibly by coupling the 2nd cysteine residues of two subunits, or by using one of the other cysteine residues in VPF_{165}. As yet we have no data to explain this dimerization satisfactorily. The fact that this mutant does not show the residual 20% activity in the mitotic assay expected from its dimerization level suggests that the "illegitimate" dimerization of mutant C4S leads to a dimer structure that is different from normal. It also does not compete for receptor binding more efficiently than mutant C2S, while the partially dimeric VPF mutant C3S does compete efficiently. In the vascular permeability assay, on the other hand, the dimeric portion of mutant C4S appears to contribute to its activity. It is an interesting hypothesis that induction of mitosis and induction of vascular permeability may involve

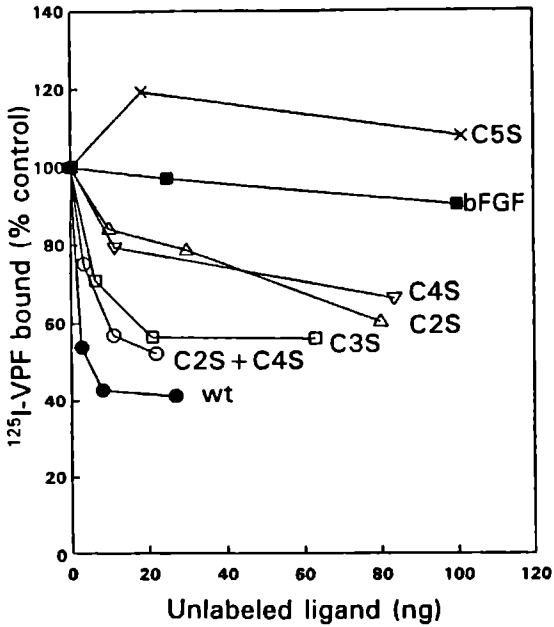

Figure 8. Competition for receptor binding by VPF mutants. ^{125}I-VPF was added to endothelial cells at $1.3x10^5$ cpm/well along with varying concentrations of unlabelled VPF (mutants) or bFGF, and binding of labelled protein to the cells was measured. See "Materials and Methods" for further details. ●: wild type VPF; ○: coexpressed VPF mutants C2S and C4S; ▵: mutant C2S; □: mutant C3S; ▿: mutant C4S; x: mutant C5S; ■: bFGF. In pBPV supernatant 1335 cpm ^{125}I-VPF (+/- 74, SD) was bound, and this value was designated as 100%. For clarity error bars were omitted; SDs were in general less than 10%.

different signal transduction pathways, one of which requires normally arranged dimeric VPF, whereas for the other an illegitimately formed S-linked dimer is sufficient. For other growth factors, like acidic fibroblast growth factor (aFGF) and Schwannoma-derived growth factor, alternative signal transduction pathways have recently been demonstrated to mediate the induction of DNA synthesis and other biological effects (35,36). Alternatively, the 50-fold difference in the concentration ranges at which the mitotic assay and the vascular permeability were carried out, may have had an influence on the non-covalent dimeric state of mutant C4S, which may also explain its differential behaviour in both assays.

It has been shown that PDGF-B mutants lacking cysteines 2 and 4 are still dimeric, albeit non-covalently bound, and fully biologically active (20,22). For VPF mutants lacking either cysteine 2 or cysteine 4 this does not hold true: the mutants are barely active in inducing endothelial cell proliferation even at high concentrations, and compete very poorly for receptor binding. Heterodimers formed upon coexpression of these two mutants have full biological activity, indicating that the subunits of mutants C2S and C4S are folded correctly. The poor ability to compete for receptor binding and the poor biological activity of mutants C2S and C4S individually is therefore caused by the mere fact that they cannot form covalent dimers linked between Cys2 and Cys4. The one interchain disulfide bridge that is formed in the C2S/C4S heterodimer obviously is sufficient to restore biological activity completely. Cross-linking data show that the

subunits of at least mutant C2S can associate non-covalently, but these interactions do not appear to contribute significantly to biological activity: apparently the non-covalent association is of a transient nature and insufficiently stable for receptor activation.

Substitution of cysteine residue 3 or 5 in VPF by a serine also led to an impaired dimerization; mutation C5S did so to a greater extent than mutation C3S. These defects could not be complemented by cotransfection with other mutants. Although the possibility cannot be excluded that these cysteines are involved in inter-subunit interactions, we suggest that cysteine residues 3 and 5 are involved in intramolecular folding, as they are in PDGF. Since Cys3 is only separated by 2 amino acids from Cys4, and Cys5 is located directly adjacent to Cys4, it is easy to imagine that loss of an intramolecular disulfide bridge in which either Cys3 or Cys5 is involved can change the position of Cys4, thus hampering the formation of the Cys2-Cys4 disulfide bridge. Mutant C5S does not even appear to form non-covalently linked dimers, suggestive for improper folding of the C5S subunits.

The hypothesis that VPF dimerization is required for receptor binding and activation is further supported by the finding that mutant C3S, which is 50% dimeric, competes for receptor binding and has a biological activity only slightly less than wild type VPF. Also consistent with this idea is the fact that mutant C5S, which does not produce detectable amounts of S-linked dimer, gives no evidence for receptor binding and has very low, if any, biological activity. Especially for mutant C5S it is also possible, of course, that the mutation of Cys5 results in an altered subunit structure which is not recognized by the receptor, even if this mutant were a dimer.

In VPF, Cys3 was not essential for biological activity, whereas activity of the VPF mutant lacking Cys5 was diminished to nearly zero. This is in contrast with the situation in the v-*sis* protein, in which Cys5 was dispensable and Cys3 was essential for transforming activity (26,27). Clearly, there must be folding differences between v-*sis* (PDGF) and VPF.

As VPF is thought to be important in tumour angiogenesis (18,19), discovery or design of VPF inhibitors might be of clinical importance. To test if any of the VPF mutants could act as a receptor antagonist, we determined if the VPF mutants with little biological activity: i.e. C2S, C4S, and C5S, were capable of inhibiting biological activity of wild type VPF. They did not, which is in accordance with the finding that they are inefficient in competing for receptor binding with labelled VPF. VPF antagonists should efficiently bind to VPF receptors without activating these receptors. Our data show that it is necessary to have VPF dimers to meet the first criterion. A strategy for designing dominant-negative VPF analogues, which dimerize with and inactivate wild-type VPF subunits, may provide a future approach towards VPF inhibition. In a similar fashion, a dominant-negative PDGF analogue (PDGF-0) has been designed recently (37).

The differential efficiency of VPF mutant C4S in inducing endothelial cell mitosis and vascular permeability, although used at different concentrations, shows that VPF variants may exist which have a partial spectrum of activities. This possibility is not only a

challenge to further study the signalling pathways involved in the different biological effects of VPF, it might also have clinical applications.

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Summary and General Discussion

Every living tissue in higher organisms is dependent on the supply of nutrients and oxygen for its survival. For this purpose nearly all mammalian tissues are equipped with and connected by a network of blood vessels. Normally, blood vessels do not grow. However, in regenerating tissues like the female reproductive organs or wounds, and in growing tissues, like embryonic and fetal tissues or tumours, the development of the vascular system has to keep pace with the development of the tissue it feeds. The growth of new blood vessels from existing ones is called angiogenesis, and is mediated by a number of factors referred to as angiogenic factors. Of these angiogenic factors, vascular permeability factor (VPF), also called vascular endothelial growth factor (VEGF), is considered to be one of the most important.

Most angiogenic factors factors stimulate the division of endothelial cells, the cells forming the inner lining of blood vessels. Apart from the direct stimulation of blood vessel growth, many angiogenic factors also induce other effects. VPF, for instance, enhances the permeability of blood vessels for proteins, and induces the expression of a factor that initiates blood coagulation, tissue factor (TF). Such effects not only contribute indirectly to the process of angiogenesis, they also lead to other interesting phenomena. For instance, a feature often observed in tumour blood vessels, is the occurrence of intravascular clotting, and obstruction of tumour blood flow by microthrombi. In some cases, like after treatment with the cytokine TNF, this can even lead to necrosis and remission of a tumour. The induction of intravascular coagulation in tumours may, in part, be mediated by tumour-secreted VPF. It is, therefore, of great interest to study VPF expression in tumours, not only because of its angiogenic property, which is beneficial for the growth of a tumour, but also because of its capacity to induce intravascular coagulation, which might be useful in anti-tumour therapy.

The initiation of intravascular clotting by VPF, or by other tumour-secreted factors, is caused by the induction of TF expression by endothelial cells, as stated above. The elevated TF expression is due to an increase in TF mRNA levels. Thus, TF induction in endothelial cells can be monitored on the level of the TF messenger. Measuring TF mRNA levels has the advantage that the mRNA can specifically be converted into, and amplified as a DNA fragment, by a technique called RT-PCR. This technique is very sensitive and thus applicable even when very small amounts of material are available. This approach is worked out in Chapter 2. A problem with quantitative RT-PCR is that corrections need to be made for tube-to-tube differences in the efficiency of amplification. We therefore designed a control RNA which was added to all reactions in an equal amount. The control RNA and the TF mRNA were co-amplified in the RT-PCR reactions, but the control RNA was chosen such that the length of its product differed from the length of the TF mRNA product. In Chapter 2 it is demonstrated that the initial

amount of TF mRNA can be estimated quite accurately from the ratio of the two products if the initial amount of control RNA is known. Furthermore, in an application of this technique, it was confirmed that the induction of endothelial cell TF activity by an unidentified melanoma cell-derived factor is accompanied by an elevation of the TF mRNA level.

Quantitative RT-PCR assays are used for the determination of messenger levels for other factors as well. Such assays may become especially useful in diagnosis if transcript levels are to be measured in very small tissue or tumour samples obtained by biopsy. Assays to determine mRNA levels for the angiogenic factors VPF and bFGF in samples of tumour material are currently being developed, using an approach similar to that for the determination of TF mRNA levels.

To study the role of VPF in tumour angiogenesis, and the correlation between VPF expression and tumour metastasis, a panel of human melanoma cell lines was used. The cells from these lines develop tumours with different biological behaviour after injection into nude mice. Some form tumours that metastasize more rapidly and frequently than the tumours from other lines. Since metastasizing tumour cells are dependent on blood vessels for their dispersion, the degree of tumour vascularization may be a rate-limiting factor in metastasis. VPF, being an angiogenic factor, might therefore determine not only the density of blood vessels in these tumours, but might also be indirectly responsible for differences in metastatic behaviour.

Studies presented in Chapter 3 demonstrated that the tumours derived from various melanoma lines had about equal levels of VPF expression. Differences in VPF expression were found between melanoma cell lines in culture: the lines which developed into the most aggressive tumours in mice, showed much higher levels of VPF expression than less malignant lines. The latter lines thus had VPF expression levels that were initially low, but which were elevated when the lines formed a tumour. This upregulation of VPF expression might be caused by low oxygen tension, as oxygen withdrawal induced enhanced VPF mRNA levels *in vitro*. Tumours often show necrotic areas, which presumably develop after a local insufficiency of blood supply, and hence of nutrients and oxygen. Upregulation of VPF expression could in that case be a direct way of improving the tumour vasculature around such an area.

To test if the expression pattern of VPF actually influences the vascular development, one such melanoma line with an inducible VPF expression was genetically altered to a constitutively expressing line by transfecting it with a VPF expression construct, and the biological effect *in vivo* was studied.

The vascular patterns in the tumours resulting from these lines were indeed dramatically different. As is shown in Chapters 3 and 4, the tumours from the parental melanoma line had many separate blood vessels penetrating the tumours in an apparently random fashion. The vessels in the VPF-overproducing tumours, on the other hand, were localized mainly in stromal septa separating tumour cell nests. These experiments have unambiguously shown that the pattern or the level of VPF expression does have an influence on the vascular development in these tumours, although it is still unclear in what way VPF

changes the vascular pattern. The blood vessels in the VPF-overproducing tumours were also hyperpermeable to proteins, which was not surprising as VPF was already known to enhance vascular permeability. The enhanced permeability of tumour blood vessels might have important implications for tumour vessel development if this would lead to a more extensive extravascular protein matrix, e.g. consisting of fibrin. Such a matrix was, however, not demonstrated.

The metastatic potential of the tumours might also be influenced by the alteration in vascular pattern. However, a significant difference between the metastatic frequencies of the tumours from the parental melanoma line and the VPF-transfected lines could not be demonstrated. The density of blood vessels in the tumours was perhaps not rate-limiting for the process of metastasis. Furthermore, the vascular phenotype in the VPF-overproducing tumours did not allow an accurate determination of the density of functional blood vessels, and this density may not have differed significantly from that in the parental tumour type. The separation between tumour cells and the blood vessels appeared to be better in the VPF-transfected tumours, however, since in these tumours the vessels were harboured within a stromal compartment, and surrounded by a broad layer of matrix proteins. The latter observation suggests that metastasis may be inhibited in the transfectant tumours rather than enhanced, but the data on the metastatic capacity of the tumours do not support this idea either.

In future experiments melanoma lines should be looked for which do not upregulate their VPF expression *in vivo* and besides have a poor vasculature. Transfecting such lines with VPF expression constructs may actually increase the density of blood vessels and enhance the growth of the tumours. It would yield a model with which the influence of tumour blood vessel density on the frequency of metastasis can be studied. Melanoma cell lines producing very slowly growing tumours, which were not included in former experiments just because of this practical disadvantage, are potential candidates for these future transfection experiments.

In Chapter 5 a study on the structure-function relationship of the VPF protein is described. As a model, the structure of PDGF was used. PDGF is a growth factor with which VPF shares 8 cysteine residues. It is a dimeric protein just like VPF, but much better characterized than VPF. In PDGF, six of the cysteine residues form intramolecular disulfide bridges, stabilizing the structure of the single PDGF chains. Two other cysteine residues form interchain disulfide bridges, which stabilize PDGF dimers: cysteine 2 of one chain is coupled to cysteine 4 of the other chain, and vice versa. It has been shown that point mutations changing either cysteine 2 or 4 of PDGF into a serine residue prevent the formation of covalently linked PDGF dimers. Coexpression of the two mutants restored the ability to dimerize. Similar cysteine to serine mutations were generated in the VPF coding sequence (see Chapter 5). The effect of these mutations on VPF dimerization were essentially as expected: single mutants lacking cysteine 2 or 4 were impaired in their ability to form covalently linked dimers, while upon coexpression the two mutants complemented each other. VPF thus is dependent on the same intermolecular disulfide bonds as PDGF for covalent dimerization.

The monomeric VPF mutants bound inefficiently to VPF receptors on endothelial cells, and had very low, if any, biological activity. The heterodimeric protein formed after coexpression of the cysteine 2 and 4 mutants, however, showed normal receptor binding and biological activity. The results thus indicated that dimerization of the VPF protein is a prerequisite for efficient receptor binding and activation. In contrast with our findings with VPF, one of the PDGF variants, the PDGF-B chain, was not dependent on covalent dimerization for biological activity. PDGF-B chains were able to form stable non-covalently linked dimers in the absence of cysteine residues 2 and 4.

Future studies might focus on the identification of receptor-binding domains of VPF, or on the design of VPF mutants that inhibit the action of the wild type protein. It should be kept in mind that dimerization is essential for effective receptor binding. For instance, peptides based on parts of the VPF amino acid sequence to be tested for receptor binding might bind only if they are cross-linked. Also, VPF mutant chains lacking the receptor-binding domain(s) might inhibit wild type VPF activity in a dominant-negative fashion if mutant and wild type chains are covalently linked.

In conclusion, the studies presented in this thesis have contributed to the understanding of the importance of VPF in the development of a tumour vascular bed. They have also provided some insight into the mechanisms of VPF dimerization and the importance of dimerization for biological activity. The outcome of these studies will hopefully be useful starting-points for future investigators on this subject.

Samenvatting en Algemene Discussie

Weefsels in hogere organismen hebben een voedsel- en zuurstofvoorziening nodig om te overleven. Daarom zijn bijna alle weefsels in zoogdieren voorzien van, en verbonden door, een netwerk van bloedvaten. Meestal is het niet nodig dat bloedvaten groeien. Soms moet het vaatstelsel echter meegroeien met het weefsel dat het van voeding voorziet, zoals in herstellende weefsels als de vrouwelijke voortplantingsorganen of wonden. Ook in groeiende weefsels, zoals in embryo's en foetussen, of in tumoren, moet het vaatstelsel zich snel ontwikkelen om de groei van het omliggende weefsel bij te houden. Het ontstaan van nieuwe bloedvaten vanuit bestaande vaten wordt angiogenese genoemd. Angiogenese wordt in gang gezet door een aantal stoffen die men angiogene factoren noemt. Vascular permeability factor (VPF), ook wel vascular endothelial growth factor (VEGF) genoemd, wordt beschouwd als een van de belangrijkste angiogene factoren.

De meeste angiogene factoren stimuleren de deling van endotheelcellen, de cellen die de binnenwand van bloedvaten vormen. Daarnaast kunnen angiogene factoren ook andere effecten teweeg brengen. VPF verhoogt bijvoorbeeld de doorlaatbaarheid van bloedvaten voor eiwitten. VPF induceert ook de expressie van een factor die bloedstolling op gang brengt, "tissue factor" (TF). Zulke "bijwerkingen" blijken vaak indirect bij te dragen aan de angiogenese, maar leiden ook tot op zich interessante fenomenen. Men neemt bijvoorbeeld vaak waar dat stolling optreedt binnen de bloedvaatjes die door een tumor lopen en dat de stolsels de stroming van het bloed door de tumor belemmeren. Wanneer een tumor wordt behandeld met het cytokine TNF kan de stolling in de tumorvaten toenemen, en kan er zelfs afsterving (necrose) van tumorweefsel en een afname van de tumorgrootte (remissie) optreden. De inductie van bloedstolling in de tumorvaten zou ook door VPF uit tumorcellen kunnen worden veroorzaakt. Het is dus van groot belang om de expressie en de rol van VPF in tumoren te bestuderen. Immers, VPF heeft een angiogene activiteit, die in het voordeel van de tumor werkt, maar kan tevens de bloedstolling op gang te brengen, wat bij anti-tumor therapie van pas zou kunnen komen.

VPF of andere factoren stimuleren de bloedstolling binnen bloedvaatjes door de TF expressie te verhogen in endotheelcellen, zoals al eerder is vermeld. Deze verhoging van TF expressie blijkt het gevolg te zijn van een toename van de hoeveelheid boodschapper RNA (mRNA) dat voor het TF eiwit codeert. Wil men de inductie van TF in endotheelcellen bestuderen, dan kan men dus ook de hoeveelheid TF mRNA meten. Deze aanpak biedt een belangrijk voordeel als er slechts weinig materiaal voorhanden is. Met de zogenaamde RT-PCR techniek kan het TF mRNA namelijk worden omgezet in een DNA molecuul, dat weer kan worden vermenigvuldigd. Wanneer echter met RT-PCR kwantitatieve metingen moeten worden gedaan, dan leveren verschillen in reactie-efficiëntie een probleem op. Dit kan worden opgelost door gelijke hoeveelheden van een controle RNA molecuul bij alle reacties te voegen, waarbij het controle RNA een

reactieproduct van een andere lengte oplevert. In Hoofdstuk 2 wordt deze aanpak uitgewerkt. Hier bleek dat het mogelijk is om de uitgangshoeveelheid TF mRNA vrij nauwkeurig te bepalen uit de verhouding van de twee RT-PCR producten, mits de uitgangshoeveelheid controle RNA bekend is. Bij de inductie van TF activiteit door een onbekende, door melanoomcellen geproduceerde factor, werd deze techniek toegepast om ook de boodschapper niveaus te meten. Daarbij bleek inderdaad dat de verhoging van TF activiteit gepaard ging met een verhoging van de hoeveelheid TF mRNA.

Kwantitatieve RT-PCR assays kunnen ook worden gebruikt voor de bepaling van mRNA niveaus van andere factoren. Voor de diagnostiek moeten vaak expressieniveaus worden gemeten in zeer kleine weefsel- of tumormonsters. Hier kunnen RT-PCR assays goed van pas komen. Er worden op dit moment assays opgezet om de mRNA hoeveelheden van de angiogene factoren VPF en bFGF te meten in tumormonsters, waarbij een soortgelijke aanpak wordt gebruikt als bij de TF mRNA bepaling in Hoofdstuk 2.

Het grootste deel van het in dit proefschrift beschreven onderzoek was er op gericht om de rol van VPF in de tumorangiogenese te bestuderen, en om een eventuele rol van VPF in de uitzaaiing (metastasering) van tumoren vast te stellen. Daarbij werd een serie humane melanoomlijnen gebruikt. Sommige lijnen vormen in naakte muizen tumoren die sneller en vaker uitzaaien naar de longen dan tumoren van andere lijnen. Omdat voor het uitzaaien van tumorcellen bloedvaten nodig zijn, is het mogelijk dat de dichtheid van bloedvaatjes in een tumor een beperkende factor is voor de metastasering. VPF zou als angiogene factor de dichtheid van tumorvaatjes kunnen beïnvloeden en tevens voor verschillen in metastaserend gedrag kunnen zorgen.

Het werk dat in Hoofdstuk 3 beschreven staat, laat zien dat VPF in ongeveer gelijke hoeveelheden geproduceerd werd in de tumoren die uit de verschillende melanoomlijnen ontstonden. Wel werden verschillen in VPF expressie waargenomen tussen de melanoomlijnen onder weefselkweekcondities. De lijnen die in muizen de meest aggressieve tumoren vormen produceren in celkweek meer VPF dan de minder kwaadaardige lijnen. De laatstgenoemde lijnen hadden dus aanvankelijk een lage VPF expressie, die echter veel hoger werd als de cellen tot een tumor uitgroeiden. Ook bleek dat een lage zuurstofspanning de inductie van VPF expressie kan veroorzaken, omdat bij gekweekte melanoomcellen een verhoging van de VPF mRNA niveaus optrad als ze zonder zuurstof gekweekt werden. Tumoren hebben vaak necrotische gedeeltes die waarschijnlijk ontstaan nadat er plaatselijk een tekort aan bloedvaten ontstaat, en daarmee een tekort aan voedingsstoffen en zuurstof. Door het opdrijven van de VPF expressie zou dan de vaatstructuur rond zo'n tumorgedeelte kunnen worden verbeterd.

Om te kunnen testen of het expressiepatroon van VPF daadwerkelijk van invloed is op de vaatontwikkeling, werd een van de melanoomlijnen genetisch veranderd door haar met een VPF expressieconstruct te transfecteren. De oorspronkelijke lijn had een induceerbare VPF expressie, maar de stabiele transfectanten brachten VPF constitutief tot expressie. Zo werd het mogelijk om het biologisch effect van gewijzigde VPF expressie *in vivo* te bestuderen.

De tumoren die uit de getransfecteerde lijnen ontstonden hadden inderdaad een opvallend afwijkend vaatpatroon. Zoals in de Hoofdstukken 3 en 4 te zien is, werden in de tumoren uit de moederlijn veelal losliggende vaatjes aangetroffen, die op een schijnbaar willekeurige wijze door de tumor heen groeiden. In de tumoren die VPF overproduceerden lagen bloedvaatjes dicht opeen gepakt in "stromale" tussenschotten die grote groepen tumorcellen van elkaar scheidden. Deze experimenten lieten duidelijk zien dat VPF expressie van belang is voor de vaatontwikkeling in deze tumoren. Het is echter nog onduidelijk via welk mechanisme VPF het vaatpatroon beïnvloedt. Daarnaast waren de bloedvaten in de tumoren uit de getransfecteerde lijnen hyperpermeabel voor eiwitten. Dit laatste is niet zo verwonderlijk, omdat van VPF al bekend was dat het de doorlaatbaarheid van vaten verhoogt. Een verhoogde vaatpermeabiliteit kan leiden tot een uitgebreid netwerk van eiwitten zoals fibrine, dat op zijn beurt de ontwikkeling van nieuwe vaatjes weer kan stimuleren. Een uitgebreide fibrinematrix werd echter niet waargenomen.

De verandering in het vaatpatroon zou ook het metastaserend vermogen van de tumoren kunnen beïnvloeden. Er kon echter geen significant verschil aangetoond worden tussen de metastasefrequenties van tumoren uit de moederlijn en die uit de getransfecteerde lijnen. Misschien was de vaatdichtheid in de tumoren wel niet beperkend voor het uitzaaiingsproces. Verder was het moeilijk te bepalen of de dichtheid van vaatjes inderdaad verschilde tussen de tumoren van de oorspronkelijke lijn en de getransfecteerde lijnen. In de tumoren van de VPF overproducerende lijnen leek de scheiding tussen de tumorcellen en de bloedvaten groter, omdat de vaatjes ingebed zaten in aparte compartimenten, omringd door een dikke laag matrixeiwitten. Dit laatste suggereert dat de metastasering in de transfectante tumoren eerder zal worden geremd dan gestimuleerd. Tot nog toe blijkt dat echter niet uit de resultaten van de metastaseproeven.

In de toekomst zou een melanoomlijn moeten worden gezocht waarbij de VPF expressie *in vivo* laag blijft, en die bovendien slecht gevasculariseerde tumoren oplevert. Na transfectie van zo'n lijn met een VPF expresieconstruct is de kans aanwezig dat de dichtheid van tumorvaatjes daadwerkelijk groter wordt en dat de tumoren sneller groeien. Zo zou een model ontstaan dat zich goed leent voor een onderzoek naar de invloed van de vaatdichtheid in tumoren op de frequentie van uitzaaiing. Er bestaan melanoomlijnen die zeer traag groeiende tumoren opleveren, en die juist vanwege dit practische probleem buiten het onderzoek naar VPF expressie zijn gehouden. Toch zijn zulke lijnen misschien het meest geschikt om het bovengenoemde experiment mee uit te voeren.

In Hoofdstuk 5 wordt een onderzoek beschreven naar de structuur-functie relatie van het VPF eiwit. Er is bij het opzetten van deze experimenten uitgegaan van de reeds bekende eiwitstructuur van PDGF. PDGF is een groeifactor die 8 cysteïnes gemeenschappelijk heeft met VPF, en die net als VPF een dimeer is. In PDGF zijn 6 cysteïnes betrokken bij intramoleculaire zwavelbruggen die de vouwing van de PDGF ketens stabiliseren. Daarnaast vormen twee andere cysteïnes zwavelbruggen met cysteïnes in een andere keten. Het tweede cysteïne van de ene keten zit gekoppeld aan het vierde cysteïne van de andere keten, en andersom. Op deze manier wordt in PDGF de dimeer gestabiliseerd.

Eerdere experimenten lieten zien dat er geen covalent gebonden dimeren konden ontstaan wanneer cysteïne 2 of 4 van PDGF vervangen werd door een serine. Werden evenwel beide mutanten tegelijk tot expressie gebracht, dan complementeerden de mutanten elkaar, en werd het vermogen tot dimerisatie hersteld. Soortgelijke mutaties werden ook in de coderende volgorde voor VPF aangebracht (zie Hoofdstuk 5). Het effect van deze mutaties op de VPF dimerisatie kwam grotendeels overeen met de verwachtingen. De mutanten waarbij cysteïne 2 of 4 door een serine was vervangen, waren hun vermogen om covalente dimeren te vormen nagenoeg kwijt, terwijl deze deficiëntie werd opgeheven bij gelijktijdige expressie van beide mutanten. VPF en PDGF bleken dus dezelfde zwavelbruggen te gebruiken voor covalente dimerisatie.

Ook bleek dat de monomere VPF mutanten inefficiënt bonden aan VPF receptoren op endotheelcellen, en dat ze een lage, dan wel geen biologische activiteit bezaten. De heterodimeren die ontstonden bij coëxpressie van de cysteïne 2 en 4 mutanten bonden echter normaal aan de receptoren en waren biologisch actief. Zo werd aangetoond dat dimerisatie van VPF een voorwaarde is voor efficiënte binding aan de receptoren en voor activering van de receptoren. Dit is heel anders dan bij de PDGF-B keten, een van de varianten van PDGF, die geen covalente dimerisatie nodig heeft voor biologische activiteit. PDGF-B ketens blijken namelijk stabiele niet-covalent gebonden dimeren te vormen, ook als de betrokken cysteines afwezig zijn.

Toekomstig onderzoek aan VPF zou zich kunnen richten op de identificatie van receptorbindende domeinen van dit eiwit, en op het ontwikkelen van VPF mutanten die de activiteit van het wildtype eiwit remmen. Hierbij moet dan de noodzaak van dimerisatie voor de receptorbinding van VPF in het oog gehouden worden. Wanneer bijvoorbeeld peptides die gebaseerd zijn op gedeeltes van het VPF eiwit, getest gaan worden op receptorbinding, dan zou het nodig kunnen zijn deze peptides twee aan twee te koppelen. VPF mutanten zonder receptorbindende domeinen zouden de activiteit van het VPF wildtype eiwit kunnen remmen op dominant-negatieve wijze, als de mutante ketens covalent worden gekoppeld aan normale ketens.

Het onderzoek dat in dit proefschrift beschreven staat heeft bijgedragen tot het begrijpen van de rol die VPF speelt in de ontwikkeling van het tumorvaatbed. Verder is er enig inzicht verschaft in de manier waarop VPF dimeriseert, en in het belang van dimerisatie voor de activiteit van VPF. De resultaten van dit onderzoek zijn hopelijk goede uitgangspunten voor onderzoekers die zich in de toekomst met deze onderwerpen zullen bezighouden.

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cDNA	complementary DNA
dATP	deoxyadenosine 5'-triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxyribonucleotides
DSS	disuccinimidyl suberate
DTT	dithiothreitol
EC	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
flk-1	fetal liver kinase-1
flt	fms-like tyrosine kinase
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HUVEC	human umbilical vein endothelial cells
IFN(α,γ)	interferon (α,γ)
IL(-1)	interleukin (-1)
IP3	inositol triphosphate
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase(pair)s
kD	kiloDalton
KDR	kinase insert domain-containing receptor
LPS	lipopolysaccharide
mRNA	messenger RNA
NCS	newborn calf serum
NTPs	ribonucleotides
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-ECGF	platelet-derived endothelial cell growth factor
PDGF	platelet-derived growth factor
PlGF	placenta growth factor
PMA	phorbol 12-myristate 13-acetate
Po_2	partial oxygen pressure
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	ribonuclease inhibitor

RT	reverse transcriptase
RT-PCR	reverse transcription-preceded PCR
SD	standard deviation
SDS	sodium dodecyl sulphate
SSC	standard saline citrate buffer
TBE	tris-borate-EDTA buffer
TF	tissue factor
TGF(α,β)	transforming growth factor (α,β)
TNF(α)	tumour necrosis factor (α)
tPA	tissue plasminogen activator
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
vWF	von Willebrand factor

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Pötgens AJG, Lubsen NH, van Altena G, Schoenmakers JGG, Ruiter DJ, de Waal RMW: Measurement of Tissue Factor Messenger RNA Levels in Human Endothelial Cells by a Quantitative RT-PCR Assay. **Thromb Haemost** 1994, 71:208-213

Pötgens AJG, Lubsen NH, van Altena MC, Vermeulen R, Bakker A, Schoenmakers JGG, Ruiter DJ, de Waal RMW: Covalent Dimerization of Vascular Permeability Factor/Vascular Endothelial Growth Factor is Essential for its Biological Activity - Evidence from Cys to Ser Mutations. **J Biol Chem** 1994, 269:32879-32885

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Curriculum vitae

Andy Pötgens werd op 5 mei 1963 geboren in Chèvremont, Kerkrade. In Kerkrade volgde hij ook de lagere en de middelbare school. In 1981 behaalde hij met lof zijn Atheneum-B diploma aan het Antonius Doctor College, en in datzelfde jaar begon hij met de studie Biologie aan de Katholieke Universiteit Nijmegen (KUN). In 1984 slaagde hij voor het Kandidaatsexamen Biologie met tweede hoofdrichting Scheikunde (B4). Vervolgens behaalde hij het diploma Deskundigheid Stralingshygiëne niveau 3, en deed hij een aantal onderzoeksstages. Als hoofdvak werd Chemische Cytologie gevolgd (Dr. F. Wanka, Dr. R. Opstelten), en als bijvakken Genetica (Prof. W. Hennig) en Toxicologie (Dr. J. Copius-Peereboom, Ir. C. Evelo). In juni 1988 slaagde hij voor het Doctoraalexamen Biologie.

Van augustus 1988 tot maart 1990 vervulde hij vervangende dienstplicht als assistent bij wetenschappelijk onderzoek in de vakgroep Moleculaire en Ontwikkelingsgenetica van de KUN (Prof. W. Hennig). Van juni 1990 tot juni 1994 is hij werkzaam geweest als wetenschappelijk medewerker op het instituut voor Pathologische Anatomie van het Academisch Ziekenhuis Nijmegen (Prof. D.J. Ruiter en Dr. R.M.W. de Waal), in een samenwerkingsproject met de afdeling Moleculaire Biologie van de KUN (Prof. J.G.G. Schoenmakers, Dr. N.H. Lubsen). Dit project werd financieel mogelijk gemaakt door de Nederlandse Kankerbestrijding (NKB). In deze periode werd het in dit proefschrift beschreven onderzoek gedaan. Tevens leverde hij een bijdrage aan tweedejaars cursussen Moleculaire Biologie, en begeleidde hij een aantal doctoraalstudenten tijdens hun onderzoeksstage Moleculaire Biologie.

Sinds januari 1995 werkt hij als post-doc op de afdeling Experimentele Immunohematologie van het Centraal Laboratorium van de Bloedtransfusiedienst (CLB) te Amsterdam.

STELLINGEN

behorende bij het proefschrift

**Vascular permeability factor,
a key regulator of endothelial cell function
and tumour angiogenesis**

Andy Pötgens, 4 mei 1995

-1-

Voor de dichtheid van tumorvaatjes, en voor de metastasefrequentie van de tumoren die in naakte muizen ontstaan uit de humane melanoomlijn Mel57, is het expressieniveau van VPF geen beperkende factor (dit proefschrift, hoofdstukken 3 en 4).

-2-

De mate van homogeniteit van de VPF expressie binnen een tumor zou wel eens een betere maat voor maligniteit kunnen zijn dan het niveau van VPF expressie (dit proefschrift, hoofdstuk 3).

-3-

De groeifactor VPF kan slechts in dimere toestand stabiel aan zijn receptoren binden (dit proefschrift, hoofdstuk 5).

-4-

Ondanks het feit dat VPF_{121} de meest voorkomende variant van VPF is in zeer veel celtypen, werd bij het onderzoek naar cellulaire bindingsplaatsen voor VPF tot nog toe steevast VPF_{165} gebruikt. Deze onderwaardering van VPF_{121} die, in tegenstelling tot VPF_{165}, een lage affiniteit voor heparine heeft, kan leiden tot te algemene conclusies omtrent de rol van heparine in de interactie van VPF met zijn receptoren.

-5-

De identificatie van hoog-affiene VPF-bindingsplaatsen op melanoomcellen, zonder dat daarbij is getest of VPF enig biologisch effect heeft op deze cellen (H Gitay-Goren et al, Biochem Biophys Res Comm 1993, 190:702-709), is vooralsnog geen reden om een autocrien mechanisme te vermoeden achter door melanoomcellen geproduceerd VPF.

-6-

De mogelijkheid om de groeifactor voor rode bloedcellen, erythropoietine (EPO), in spierweefsel tot expressie te brengen na intramusculaire inspuiting van adenovirussen met het voor dit eiwit coderende DNA (SK Tripathy et al, Proc Natl Acad Sci USA 1994, 91:11557-11561), doet vrezen dat "gendoping" in de toekomst praktijk zal worden in de topsport.

-7-

Als wetenschappers bij het interpreteren van hun eigen onderzoeksresultaten dezelfde scepsis aan de dag zouden leggen als bij het refereren van andermans manuscripten, zou het aantal publicaties aanzienlijk lager liggen dan de 535.000 die per jaar alleen al op het gebied van de biologie worden geregistreerd (gegevens BIOSIS Previews 1990).

-8-

Het gesleep met gigantische hoeveelheden voedsel vanuit ontwikkelingslanden ten behoeve van de Nederlandse veeteelt, veroorzaakt hier en in de productielanden milieuproblemen, en zal er uiteindelijk voor zorgen dat heel Nederland boven de zeespiegel komt te liggen.

-9-

Analyse van de recente voorspellingen door Rijkswaterstaat van de te verwachten waterstanden in de grote rivieren leert dat van het water vaak geen hoogte is te krijgen.

-10-

Een Limburger die vanwege de werkgelegenheid afzakt naar de Randstad is een zuivere economische vluchteling.

-11-

De beste wetenschapper blijft altijd een beginneling.

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